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(54) Title: DYES AND METHODS OF DETECTION OF NUCLEIC ACID IN IMMATURE RED BLOOD CELLS

(57) Abstract: The dyes of the present invention are useful for many purposes that include markers or tags for detecting the presence of a molecule or compound to which they are bound. The dyes may be either red-excitable or blue-excitable. The dyes of the invention are particularly well suited for staining of nucleic acids. For example, these dyes are particularly suitable for staining of RNA in reticulocytes. In another exemplary application, these dyes are suitable for staining DNA in nucleated red blood cells. Typically, when used in staining of nucleic acids, the dyes are formulated into reagent solutions. In addition, the invention provides compositions and methods for facilitating rapid transport of dye molecules through a cell membrane. Such rapid staining requires that a sample be contacted with a dye composition of the invention in the presence of at least one surfactant and optionally, a sulfonic acid or a salt thereof.

# DYES AND METHODS OF DETECTION OF NUCLEIC ACID IN IMMATURE RED BLOOD CELLS

## Field of the Invention

This invention generally relates to the field of new dyes, dye compositions, and methods of detection of nucleic acids in red blood cells by flow cytometry by employing fluorescent, nucleic acid binding dyes. More particularly, this invention provides a reagent and method for enumeration of reticulocytes and nucleated red blood cells.

## Background of the Invention

Analysis of red blood cells and enumeration of its immature sub populations are a valuable components of diagnostic hematology. For example, enumeration of reticulocytes, i.e., the immature erythrocytes, in human peripheral blood is useful in the diagnoses of hemorrhage, anemia, monitoring bone marrow transplantation and for monitoring patients undergoing chemotherapy and other disorders involving blood cell production [U.S. Patent No. 5,360,739; H. Shapiro, Practical Flow Cytometry, 3rd edit., 1995; Wiley-Liss, New York; Davis *et al*, (1990) Pathobiol., 58:99-106; Hoy, (1990) Bailliere's Clin. Haemat., 3:977-988; H. J. Tanke, Reticulocytes and Mature Erythrocytes in *Flow Cytometry in Haematology* (1992) Academic Press Ltd., pp. 75-93]. Because reticulocytes contain ribonucleic acid (RNA), if stained with RNA binding excitable dyes, these cells fluoresce when illuminated by a light source of appropriate wavelength. RNA binding dyes have been used to distinguish reticulocytes from mature red blood cells (RBCs) which lack RNA.

Distribution of fluorescence intensities of a relatively large reticulocyte population can be determined by flow cytometry in a fast and reliable manner, and different maturation stages of reticulocytes, as reflected by differences in RNA content, can be distinguished.

The use of red-excitable dyes is desirable because such dyes are detected by excitation with relatively inexpensive diode or HeNe lasers. However, initial efforts in the prior art to employ diode lasers and red-excited dyes for rapid flow

cytometric analyses of reticulocytes were not successful. Yamamoto, U.S. Patent No. 5,563,070 suggested that the addition of large quantities of TO-PRO-3, a red-excitable dye, followed by a 30 minute incubation, stained RNA inside living reticulocytes. Such a method, however, is not practical for routine analysis of reticulocytes in clinical laboratories that require high sample throughput because sample preparation time is long and cost per test is high due to the large amount of dye required to stain each sample.

A red-excitable dye called Thiazole Blue (TB) has been described in U.S. Patent 4,957,870. However, as described in this patent (U.S. Patent 4,957,870), this dye also requires long periods of incubation, of about 30 minutes.

Akai et al., U.S. Patent No. 5,821,127 have also described the preparation of fluorescent dyes which are capable of detecting reticulocytes using inexpensive detectors via fluorescence in the red region. However the samples require incubation at elevated temperatures of about 40°C.

More recently, U.S. Patent No. 5,994,138, described staining reticulocytes via the use of a red-excitable dye in combination with a detergent and an ionophore at elevated temperatures of about 35°C. However, staining reticulocytes was not successful when ambient temperatures were utilized.

Fan et. al (U.S. 5,411,891, U.S. 5,360,739) describes clearly that the specific binding constant between a dye and reticulocyte RNA and the rate of penetration of the dye are different for each dye and that it is impossible to predict under what conditions a particular dye may rapidly penetrate red cell membrane and stain reticulocytes. This was further supported by Akai et. al (U.S. 5,821,127).

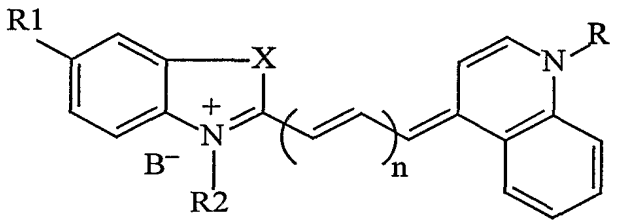
Thus, there exists a need in the art for dyes, compositions and methods which enable rapid staining of intracellular RNA at room temperatures via the use of dyes that are excitable in the red region and can use inexpensive and readily available red-illumination instruments. In addition there exists a need in the art for dye compositions and methods that are applicable not only to red-excitable dyes, but also to dyes excitable at other wavelengths, such as in the blue wavelength. By doing so, ready and accurate detection of reticulocytes can be accomplished without particular restriction of the excitation wavelength.

In addition to the reticulocytes, another type of immature red blood cells that are important in clinical diagnostics are the nucleated red blood cells (NRBCs). In contrast to reticulocytes, NRBCs contain DNA. NRBCs normally occur in the bone marrow but not in peripheral blood. However, in certain diseases such as anemia and leukemia, NRBCs also occur in peripheral blood. Therefore, it is of clinical importance to measure NRBC. Since NRBCs contain DNA, fluorescence detection and enumeration of NRBCs can be possible by staining these cells with dyes that recognize DNA. Thus, there exists a need in the art for dyes, and compositions which enable staining of both intracellular DNA and RNA, so that both NRBCs and reticulocytes can be enumerated via the use of the same dye employing different methods.

## Summary of the Invention

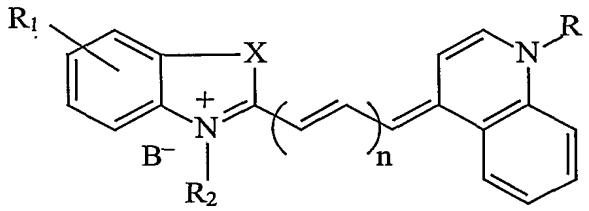
In one aspect, the present invention provides three groups of novel dyes that are set forth as Group I, Group II, and Group III with:

Group I having the general formula of:




wherein n is 0, 1, 2, or 3; $R_1$ is H, alkyl, or an alkoxy group; $R_2$ is $CH_2(CH_2)_mOH$, wherein m is 0, 1, 2, or 3; X is O, S, or $C(CH_3)_2$; R is $CH_3$, $CH(CH_3)_2$, $CH_2CH_2OH$, alkyl, alkylsulfonate, or hydroxyalkyl; and $B^-$ is a counteranion.

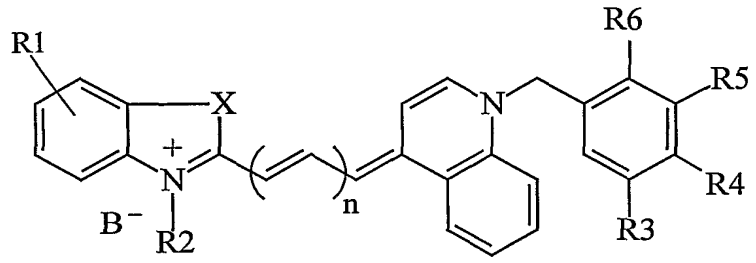
Group II having the general formula of:



wherein, n is 0, 1, 2, or 3; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOAc$, wherein m is 0, 1, 2, or 3; X is O, S, or $C(CH_3)_2$; R is $CH_3$, $CH(CH_3)_2$, $CH_2CH_2OAc$, alkyl, alkylsulfonate, or hydroxyalkyl and B- is a counteranion;

and,

Group-III having the following general formula:



wherein n is 0, 1, or 2; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOH$, or $CH_3$ ; X is O, S, or $C(CH_3)_2$; $B^-$ is a counter anion, and R3, R4, R5, R6 are various substituents as represented by the formulae for the specific compounds Dye-3 through Dye-6.

In another aspect, the invention provides a reagent containing a dye of the invention and a solvent.

In yet another aspect, the invention provides compositions and methods for facilitating rapid transport of dye molecules through a cell membrane. Such rapid staining requires that a sample be contacted with a dye composition of the

invention in the presence of at least one surfactant and optionally, a sulfonic acid or a salt thereof.

Other aspects and advantages of this invention will be readily apparent from the detailed description of the invention.

Brief Description of the Drawings

Fig. 1A is the comparison of the fluorescence spectra of the dye compound No. 6 described in example 1, hereinafter called ReticRed1, in PBS solution with or without RNA present in the solution.

Fig. 1B is the comparison of the fluorescence intensity of the dyes of this invention and several other dyes studied during this experiment, relative to the fluorescence intensity of a commercial nucleic acid dye Syto 62 (Molecular Probes, Inc), all in presence of same amount of RNA in the solution.

Fig. 1C shows the fluorescence intensities of the dyes, ReticRed1 through ReticRed6, of this invention in presence of 8 mg/ml RNA compared to their respective fluorescence intensities when there is no RNA present in the solution.

Figs. 2A and 2B show the fluorescence from reticulocytes for an abnormal blood sample which were treated with the dye ReticRed1 in an isotonic saline solution without the presence of the rapid staining composition, and incubated for two different durations. Fig. 2A shows fluorescence from cells treated with ReticRed1 alone and incubated for about 10 min: Total count 42660, Red cells 32841, Reticulocytes 801, calculated retic percentage 2.4%. Fig. 2B shows fluorescence from cells treated with ReticRed1 alone and incubated for about 40 min: Total count 46199, Red cells 39713, Reticulocytes 2310, calculated retic percentage 5.8%. The reference value for reticulocyte in this sample was approximately 7%.

Fig. 3 shows the fluorescence from reticulocytes in the blood sample, from the same donor that was used in the experiments described in Figs. 2A and 2B, after being treated with a composition of the invention containing the dye ReticRed1 and incubated for about 1 min. The percentage of reticulocytes enumerated by this method was 7.3%.

Fig. 4 shows the fluorescence from reticulocytes in an abnormal blood sample, after being treated with a composition of the invention containing the dye ReticRed1 and incubated for about 1 min. The percentage of reticulocytes enumerated by this method was 7.5%. The reference value for reticulocytes percentage obtained for this donor using an independent measurement method was 8%. The reagent composition in this example included Igepal, Dodecyl-β-D-maltoside and p-toluenesulfonic acid monohydrate.

Fig. 5 shows the fluorescence from reticulocytes in the blood sample from a normal donor, after being treated with a composition of the invention containing the dye ReticRed1 and incubated for about 1 min. The percentage of reticulocytes enumerated by this method was 0.82%. The reagent composition in this example included Igepal, Dodecyl-β-D-maltoside and p-toluenesulfonic acid monohydrate. The reference value for reticulocyte percentage obtained for this donor using an independent measurement was 0.95%.

Fig. 6 shows the fluorescence from reticulocytes in an abnormal blood sample, after being treated with a composition of the invention containing the dye ReticRed1 and incubated for about 1 min. The percentage of reticulocytes enumerated by this method was 13.7%. The reference value for reticulocyte percentage obtained for this donor using an independent measurement was 13%. The reagent composition in this embodiment included Igepal, Dodecyl-β-D-maltoside and sodium p-toluenesulfonate.

Fig. 7 shows the fluorescence from reticulocytes in an abnormal blood sample, after being treated with a composition of the invention containing the dye ReticRed3 and mixed for about 1 sec with no further incubation. The percentage of reticulocytes enumerated by this method was 13.8%. An independent reference value for reticulocyte percentage obtained for this sample by a conventional Retic-Count™ method was approximately 13.3 %. The reagent composition in this embodiment included Igepal®, Dodecyl-β-D-maltoside and sodium p-toluenesulfonate.

Fig. 8 shows the fluorescence from reticulocytes for in abnormal blood sample, after being treated with a composition of the invention containing the dye

ReticRed3 and mixed for about 1 sec with no further incubation. The percentage of reticulocytes enumerated by this method was 1%. An independent reference value for reticulocyte percentage obtained for this sample by a conventional Retic-Count™ method was approximately 1.3 %. The reagent composition in this embodiment included Igepal, Dodecyl-β-D-maltoside and sodium p-toluenesulfonate.

Fig. 9A is the comparison of the fluorescence spectra of the dye Dye-1 described in example 1B, in PBS solution with or without RNA present in the solution.

Fig. 9B is the comparison of the fluorescence spectra of the dye Dye-1 described in example 1B, in PBS solution with or without DNA present in the solution.

Fig. 9C is the comparison of the fluorescence spectra of the dye Dye-2 described in example 1B, in PBS solution with or without RNA present in the solution.

Fig. 9D is the comparison of the fluorescence spectra of the dye Dye-2 described in example 1B, in PBS solution with or without DNA present in the solution.

Fig. 9E is the comparison of the fluorescence spectra of the Dye-3 described in example 1C, in PBS solution with or without RNA present in the solution.

Fig. 9F is the comparison of the fluorescence spectra of the Dye-3 described in example 1C, in PBS solution with or without DNA present in the solution.

Fig. 9G is the comparison of the fluorescence spectra of the Dye-4 described in example 1C, in PBS solution with or without RNA present in the solution.

Fig. 9H is the comparison of the fluorescence spectra of the Dye-4 described in example 1C, in PBS solution with or without DNA present in the solution.

Fig. 9I is the comparison of the fluorescence spectra of the Dye-5 described in example 1C, in PBS solution with or without RNA present in the solution.

Fig. 9J is the comparison of the fluorescence spectra of the Dye-5 described in example 1C, in PBS solution with or without DNA present in the solution.

Fig. 9K is the comparison of the fluorescence spectra of the Dye-6 described in example 1C, in PBS solution with or without RNA present in the solution.

Fig. 9L is the comparison of the fluorescence spectra of the Dye-6 described in example 1C, in PBS solution with or without DNA present in the solution.

Figs. 10A, 10B, and 10C show the fluorescence from reticulocytes for three different blood samples (one normal, two abnormal high retics) which were treated with the dye Dye-1 in an isotonic saline solution in the presence of a rapid staining composition, and analyzed instantaneously thereafter. In these examples the reagent composition used to facilitate rapid staining included Igepal®, and Dodecyl-β-D-maltoside.

Figs. 11A, 11B, and 11C show the fluorescence from reticulocytes for three different blood samples (one normal, two abnormal high retics) which were treated with the dye Dye-2 in an isotonic saline solution in the presence of a rapid staining composition, and analyzed instantaneously thereafter. In these examples the reagent composition used to facilitate rapid staining included Igepal®, and Dodecyl-β-D-maltoside.

Figs. 12A, 12B, and 12C show the fluorescence histograms of red blood cells containing different numbers of reticulocytes. For these three different blood samples (one normal, two abnormal high retics), each sample was treated with the Dye-3 in an isotonic saline solution in the presence of a rapid staining composition, and analyzed on a BECKMAN COULTER™ XL™ flow cytometer instantaneously thereafter. In this example the reagent composition used to facilitate rapid staining included the detergent Igepal, and the surfactant Dodecyl-β-D-maltoside.

Detailed Description of the Invention

The present invention provides dyes for staining nucleic acids, as well as compositions and methods to facilitate rapid transport of dyes through cell membranes for staining reticulocytes. Advantageously, the dyes of the invention are characterized by exhibiting significantly stronger fluorescence when bound to nucleic acids than when unbound.

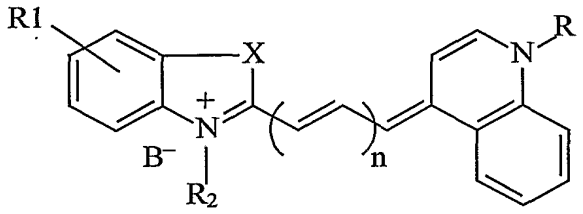
In a preferred embodiment, suitable samples containing reticulocyte cell populations for analyses using the dyes, compositions and methods of the present invention are preferably selected from whole blood, or blood samples that have been enriched or depleted by certain additional processes prior to staining. Such enrichment or depletion processes may include, among others, centrifugation, ficoll assisted gravity separation or magnetic separation.

Advantageously, the dyes, methods and compositions of the invention do not require cell fixation and are therefore particularly well suited for use in analyses of metabolically active cells. However, selection of the cell population is not a limitation on the present invention.

As discussed above, only a few dyes, which have the ability to fluoresce in the red or blue region, are suitable for reticulocyte enumeration. These dyes, however, require the use of elevated temperatures, necessitate long incubation periods, create large amounts of non-specific background fluorescence, and/or show little enhancement of fluorescence in presence of RNA. The present invention provides novel dyes, both red and blue-excitable, which bind nucleic acids and permit detection of reticulocytes via fluorescence at ambient temperatures. Suitably, the dyes of the invention are adapted to methods which preferentially permit rapid detection of reticulocytes in the absence of substantial background fluorescence. An advantage of the method of the invention is the speed at which the staining is accomplished and the fact that it can be achieved using whole blood at room temperature.

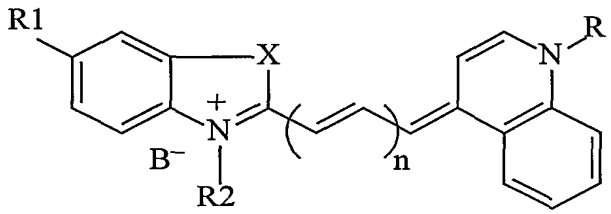
The present invention provides three groups of novel dyes that are set forth as:

Group I having the general formula of:



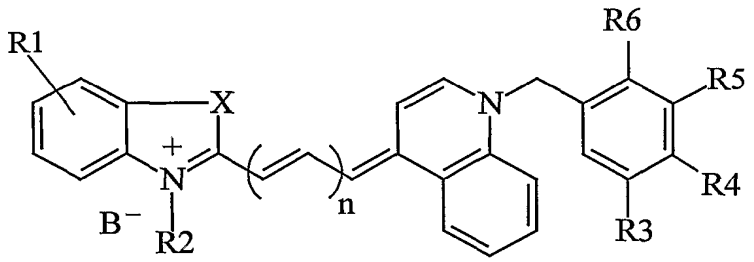

wherein n is 0, 1, 2, or 3; $R_1$ is H, alkyl, or an alkoxy group; $R_2$ is $CH_2(CH_2)_mOH$, wherein m is 0, 1, 2, or 3; X is O, S, or $C(CH_3)_2$; R is $CH_3$, $CH(CH_3)_2$, $CH_2CH_2OH$, alkyl, alkylsulfonate, or hydroxyalkyl; and $B^-$ is a counteranion.

Group II having the general formula of:




wherein, n is 0, 1, 2, or 3; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOAc$, wherein m is 0, 1, 2, or 3; X is O, S, or $C(CH_3)_2$; R is $CH_3$, $CH(CH_3)_2$, $CH_2CH_2OAc$, alkyl, alkylsulfonate, or hydroxyalkyl; and B- is a counteranion.

Group III having the general formula of:



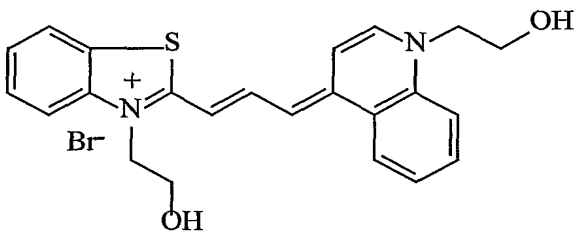

wherein n is 0, 1, or 2; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOH$, or $CH_3$ ; X is O, S, or $C(CH_3)_2$; $B^-$ is a counter anion, and R3, R4, R5, R6 are various substituents as represented by the formulae for the specific compounds Dye-3 through Dye-6.

According to the invention, a counterion includes, without limitation, a single element or a negatively charged group. In one embodiment of the invention, the counteranion is a halide selected from among $Br^-$, $Cl^-$, or $I^-$. In another embodiment of the invention, the counteranion is a tosylate group ($OTs^-$), wherein $OTs^-$ is $[CH_3(C_6H_4)SO_3]^-$. In still another embodiment of the invention, the counteranion is$BF_4^-$. However, the invention is not so limited. It is within the skill of one in the art to select a suitable counteranion, from among those known ions and ionic groups, including, without limitation, $PF_6^-$.

This invention provides dye compounds having the general formula given above, which may be either red-excitable or blue-excitable. As used herein, a red excitable dye is a dye which fluoresces when illuminated with light of a wavelength in the red spectral range. As used herein, this red spectral range is from about 600 nm to about 725 nm, and preferably, 630 nm to 670 nm. A blue excitable dye is a dye which fluoresces when contacted with light of a wavelength in the blue spectral range. As used herein, a blue excitable dye can typically have an absorption maximum in the range of 420 nm to 560 nm, and preferably from about 450 nm to about 520 nm.

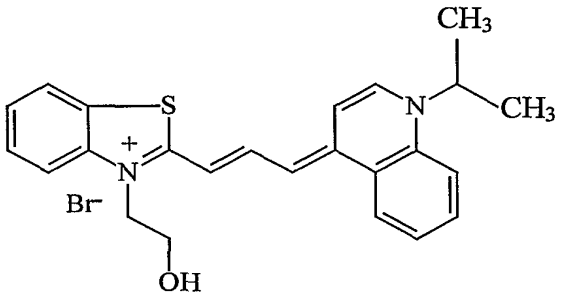
As stated above, the dyes of the invention show strong enhancement of fluorescence when bound to nucleic acids. Generally, the dyes of the Group I general formula have a fluorescence enhancement ratio of greater than about 200 (ratio of fluorescence intensity of RNA-bound dye to fluorescence intensity of free dye). In some embodiments, the dyes of the invention have a fluorescence enhancement ratio of greater than about 300. Still other dyes of the invention will have a fluorescence enhancement ratio of greater than about 350, yet other dyes of the invention will have a fluorescence enhancement ratio of greater than about 400, and still other dyes of the invention will have a have a fluorescence enhancement ratio of greater than about 450. For specific examples, see, e.g., Figure 1B and Table 1. However, the present invention is not limited by the ratios provided herein.

In one desirable embodiment, the invention provides a dye, termed herein, ReticRed1 having the following formula.



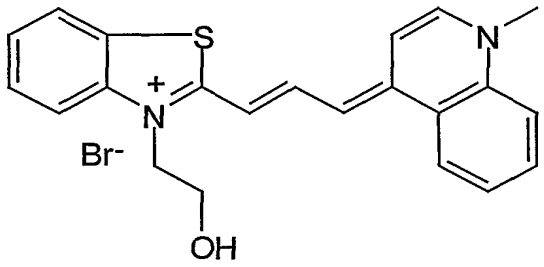

ReticRed1 is further described in Example 1A, compound (6). With reference to the general formula, in ReticRed1, n is 1, R1 is H, R2= $CH_2CH_2OH$, R=$CH_2CH_2OH$, X is S, and $B^-$ is Br-.

In another desirable embodiment, the invention provides a dye, termed ReticRed2, having the formula:



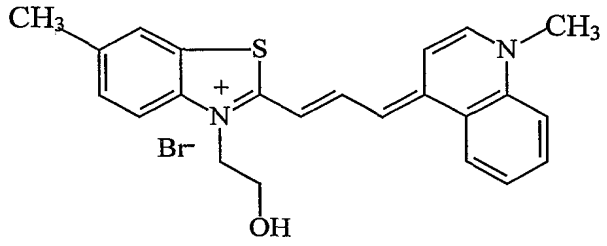

With reference to the general formula, in ReticRed2 n is 1, $R_1$ is H, R is $CH(CH_3)_2$, X is S, $R_2$ is $CH_2CH_2OH$, and $B^-$ is $Br^-$.

In yet another desirable embodiment, the invention provides a dye, termed ReticRed3, having the formula:



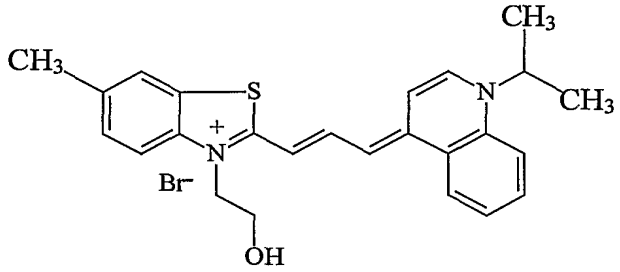

With reference to the general formula, in ReticRed3 n is 1, $R_1$ is H, R is $CH_3$, $R_2$ is $CH_2CH_2OH$, X is S, and $B^-$ is $Br^-$.

In still another desirable embodiment, the invention provides a dye, termed ReticRed4, having the formula:



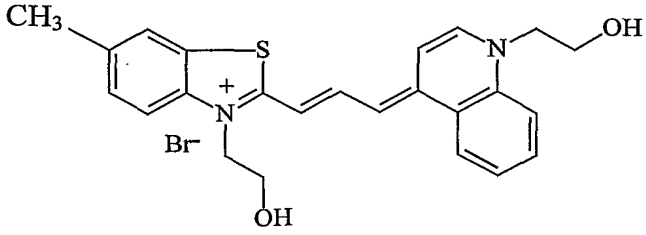
With reference to the general formula, in ReticRed4, n is 1, $R_1$ is $CH_3$, R is $CH_3$, $R_2$ is $CH_2CH_2OH$, X is S, and $B^-$ is $Br^-$.

In a further embodiment, the invention provides a dye, termed ReticRed5, having the formula:



With reference to the general formula, in ReticRed5, n is 1, $R_1$ is $CH_3$, R is $CH(CH_3)_2$, $R_2$ is $CH_2CH_2OH$, X is S, and $B^-$ is $Br^-$.

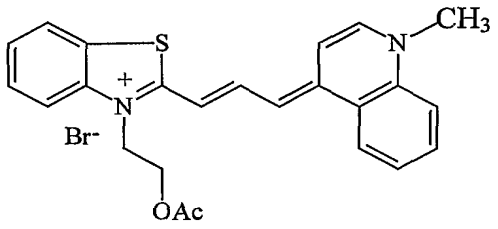
In another desirable embodiment, the invention provides a dye, termed ReticRed6, having the formula:



With reference to the general formula, in ReticRed6, n is 1, $R_1$ is $CH_3$, R is $CH_2CH_2OH$, $R_2$ is $CH_2CH_2OH$, X is S, and $B^-$ is $Br^-$.

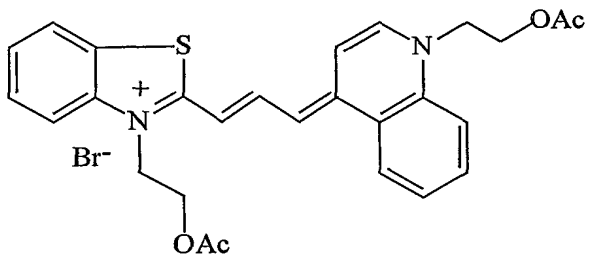
In yet another desirable embodiment, the invention provides a blue-excitable dye, termed ReticBlue1, where with reference to the general formula, n is 0. One particularly desirable example of a preferred blue excitable dye is ReticBlue1, which is represented by the general formula, wherein, n=0, R1=H, m=1, R2= $CH_2CH_2OH$, R=$CH_2CH_2OH$, X=S, $B^-$ =$Br^-$

In one desirable embodiment, the invention provides a dye, termed herein, Dye-1 having the following formula:



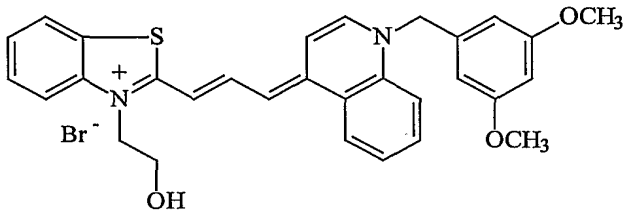

With reference to the *Group-II General Formula*, the Dye-1 has n=1; $R_1$ = H; R = $CH_3$; $R_2$ = $CH_2CH_2OAc$; X = S; and $B^-$ is $Br^-$.

In another desirable embodiment, the invention provides a dye, termed Dye-2, having the formula:



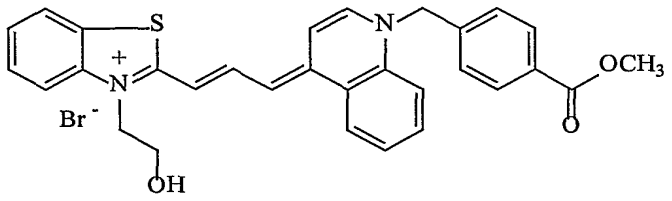
Dye-2 is further described in Example 1B. With reference to the *Group-II General Formula*, the Dye-2 has n = 1, R1 = H, R2 = R = $CH_2CH_2OAc$, X = S, and $B^-$ is Br-.

In another desirable embodiment, the invention provides a dye, termed herein, Dye-3 having the following formula:



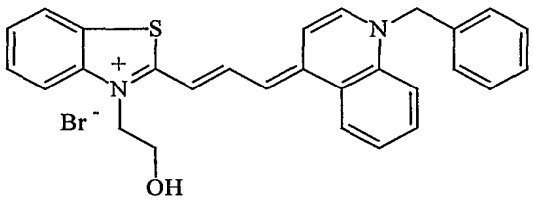

Dye-3 is further described in Example 1C. With reference to the *Group-III General Formula*, the Dye-3 has n=1, X is S, R1=H, R2= $CH_2CH_2OH$, R3 = R4 = $OCH_3$, R5=R6=H, and $B^-$ is Br-.

In another desirable embodiment, the invention provides a dye, termed Dye-4, having the formula:



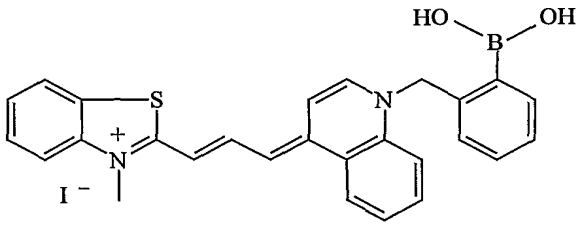

Dye-4 is further described in Example 1C. With reference to the *Group-III General Formula*, the Dye-4 has n= 1; X = S; R1=H; R2= $CH_2CH_2OH$; R3 = R5 = R6=H; R4=$CO.CH_3$; and $B^-$ is $Br^-$.

In another desirable embodiment, the invention provides a dye, termed Dye-5, having the formula:



Dye-5 is further described in Example 1C. With reference to the *Group-III General Formula*, the Dye-5 has n= 1; X = S; R1=H; R2= $CH_2CH_2OH$; R3= R4= R5= R6= H; and $B^-$ is $Br^-$.

In yet another desirable embodiment, the invention provides a dye, termed Dye-6, having the formula:



Dye-6 is further described in Example 1C. With reference to the *Group-III General Formula*, the Dye-6 has n= 1; X = S; R1=H; R2= $CH_2CH_2OH$; R3= R4= R5= H; R6= $B(OH)_2$; and $B^-$ is $I^-$.

The dyes of the invention are useful for a variety of purposes which will be readily apparent to those of skill in the art. Such uses include, for example, use of dyes as markers or tags for detecting the presence of a molecule or compound to which they are bound. Such markers may be used for monitoring the efficacy of a therapeutic compound in vivo or for diagnostic uses, or the like. However, the dyes of the invention are particularly well suited for staining of nucleic acids. For example, these dyes are particularly suitable for staining of RNA in reticulocytes. In another exemplary application, these dyes are suitable for staining DNA in nucleated red blood cells. Typically, when used in staining of nucleic acids, the dyes are formulated into reagent solutions.

## I. Staining Reagents

The invention provides a variety of compositions, described herein, which are useful as staining reagents in a variety of applications, including a variety of assay formats. Examples of such uses will be readily apparent to those of skill in the art, upon a review of the compositions described herein.

### A. *Dye Compositions*

In order to utilize the novel dyes of the invention for staining of nucleic acids, the dyes are dissolved in an appropriate solvent to form solutions. As defined herein, a solvent is meant to describe any liquid that can dissolve a solid, liquid, or gas. A preferred embodiment of the invention utilizes water-based or miscible liquids, such as dimethylsulfoxide (DMSO), methanol, ethanol, and mixtures thereof. Selection of a suitable solvent is however not a limitation on the present invention. For storage, typically a relatively high concentration of the dye is dissolved in a suitable solvent to obtain a stock solution. In a preferred embodiment, to obtain a stock solution of the dye, the dye is dissolved in DMSO at a dye concentration ranging from 0. 5 mM to 10 mM, and most preferably, 1 to 5 mM.

For further application of the dye composition in the method of the invention, the stock solution may be diluted in other reagents or buffers, including, for example, water, saline, phosphate buffered saline (PBS), or isotonic saline. The final dye concentration in such dye compositions may be varied depending on the application. In one embodiment, the concentration of the dye in the final dye composition added to a sample of whole blood is in the range of about 0.1-50 μM, preferably from about 0.5 to 25 μM, and more preferably 2 to 10 μM. However, selection of an appropriate concentration or diluting solvent is not a limitation on the present invention.

The dye composition of the invention can be utilized for a variety of purposes. A preferred embodiment of this invention is the use of the dye composition for staining, detection, and analysis of nucleic acids. Another

preferred embodiment of this invention is the use of the dye composition for staining, detection, and analysis of reticulocytes in whole blood. Yet another preferred embodiment of this invention is the use of the dye for staining, detection and analysis of nucleated red blood cells in whole blood. One of skill in the art will readily understand that use of the dyes and reagents of the present invention is not so limited. In one particularly desirable embodiment, for example, where the dye contacts intact cells, it can be desirable to formulate one or more of the dyes into a mixture.

B. *Compositions for Rapid Staining:*

In another aspect, a dye composition of the invention may be utilized to facilitate rapid transport of the dyes through the cell membrane, thereby permitting the dye to stain reticulocytes in about 1 minute or less. Thus, the method of the invention can permit staining in about 0 to about 60 seconds, preferably from about 0 seconds to about 45 seconds, more preferably from about 0 seconds to about 30 seconds, most preferably from about 0 seconds to about 20 seconds.

Generally, such rapid staining requires that a sample be contacted with a dye composition of the invention in the presence of at least one surfactant and optionally, a sulfonic acid or a salt thereof.

When used for rapid staining, additional components may be added to the composition of the invention. For example, buffers may be employed in situations where maintaining the pH of the dye composition is necessary. Preferably, the pH of the dye reagent is maintained in the range of about 6 to about 9. More preferably, a pH in the range of about 7 to about 7.5 is obtained. The buffer may be selected from a variety of buffers known to those of skill in the art to be used in the compositions of the invention and include, without limitation, phosphate buffered saline (PBS) or isotonic saline, such as ISOTON®II diluent, U.S. Patent 3,962,125, [Beckman Coulter, Inc., Miami, Florida], or the like. Additionally, such buffers may also be used to adjust the concentration of one or more of the components of the composition of this invention. Preservatives may also be added to the compositions of the invention, and may be selected from, but is not limited to, 5-Chloro-2-methyl-4-isothiazolin-3-one, and 2-methyl-4-isothiazolin-3-one

[such preservatives may be purchased commercially, e.g., as ProClin 300 or ProClin 150].

At least one surfactant is used in the rapid staining composition. In one exemplary embodiment, the surfactants include a detergent and a second surfactant which functions as a sphering agent for a cell. In another exemplary embodiment, the surfactants may be designed such that a single surfactant functions as both a detergent and a sphering agent. In yet another exemplary embodiment, the surfactants may be designed such that a single surfactant provides the necessary surfactant function, but no sphering reagent function is required.

Surfactants to be used in the composition of the invention may be selected from among the anionic surfactant ammonium perfluoralkyl carboxylate [commercially available as Fluorad® FC-143 (3M Company, Minneapolis, Minnesota)], sodium lauroyl myristoyl lactylate [commercially available as Pationic® 138C (R.I.T.A. Corp, Woodstock, Illinois)], or from the non-ionic surfactants Dodecyl- β- D-maltoside, N, N-bis[3-D-glucon-amidopropyl] cholamide, polyoxypropylene-polyoxyethylene block copolymer, N-Tetradecyl-β-D-maltoside, Daconyl-N-methyl-glucamide, n-Dodecyl-β-D-glucopyranoside, n-Decyl-β-D-glucopyranoside, polyethylene glycol ester of stearic acid, ethoxylated cocomonoglyceride, octyphenoxypoly (ethyleneoxy) ethanol, ethoxylated octylphenol, and linear alcohol, or, from among the cationic surfactants, coco hydroxyethyl imidazoline, lauryltrimethylammonium chloride, decyltrimethylammonium bromide, octyltrimethylammonium bromide, or from among the zwitterionic surfactants lauramidopropyl betaine, N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, cocoamidopropylbetaine, cocoamidosulfobetaine, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate.

As discussed above, the surfactant selected may function as a sphering agent for red cells when the concentration and osmolarity is appropriately adjusted. A sphering agent may be readily selected by one of skill in the art. A preferred sphering reagent is based on the non-ionic surfactant Dodecyl-β-D-maltoside,

which suitably is in solution with a buffer such as phosphate buffered saline. To effectively isovolumetrically sphere the reticulocytes and red blood cells within a blood sample, the concentration of the sphering reagent in the composition is most preferably from about 3 μg/ml to about 50 μg/ml with a mOsm in the range of about 200 to about 400 mOSm, and preferably from about 250 mOsm to about 350 mOsm. However, one of skill in the art may readily adjust this concentration and osmolarity as needed or desired to isovolumetrically sphere the cells, taking into consideration the surfactant selected. As discussed above, optionally, the selection of the sphering reagent may eliminate the need for the detergent.

In one embodiment, a detergent is included in the dye composition of the invention. Detergents to be used are selected from among non-ionic detergents. Desirably, these detergents are used at a concentration between about 0 to about 1%. In one preferred embodiment of the invention, the detergents are used at concentrations of between about 0.001% to about 0.5%, and in a more preferred embodiment are in the range from about 0.005% to 0.1%. One currently preferred detergent is octylphenoxypoly(ethyleneoxy)ethanol [commercially available as Igepal® CA-630 (Sigma N-6507) or Nonidet P-40 (Sigma)]. Examples of other suitable detergents include, ethyoxylated octylphenol [commercially available as Triton X-100 (Sigma T9284)], and linear alcohol alkoxylates [commercially available as Plurafac® A-38 (BASF Corp) or Plurafac® A-39 (BASF Corp)]. Typically, these detergents are mixed with a sample (or vice versa), e.g., 1-2 μl of whole blood or the like, or separately formulated into a composition of the invention.

In one desirable embodiment, a sulfonic acid, or a salt thereof, is included in the composition of the invention. The sulfonic acid or salt thereof acts as a further facilitator of transporting the dye composition through the cell membrane and is used at a concentration ranging from about 0.01 to about 250 μM, most preferably from 0.01 to about 50 μM. One embodiment of this invention utilizes p-toluenesulfonic acid. In other embodiments of this invention, a p-toluenesulfonate salt can be substituted for p-toluenesulfonic acid. Examples of such salts include sodium, potassium, silver, zinc, and barium p-toluenesulfonate

salts. Some examples of such salts are commercially available, e.g., from Sigma-Aldrich. Other suitable sulfonates may be readily selected for use in the invention.

The dye compositions of the invention can contain a single dye, optionally in the presence of other dye. In one embodiment of the invention, a composition of the invention can contain one or more of the dye compounds of this invention, or any combination thereof.

In yet another embodiment of the invention, the composition of the invention can contain other dyes known to those of skill in the art in combination with one or more of the novel dyes of the invention. Such dyes are readily selected from among dyes which have been published and/or which are commercially available. See, for example, Beckman Coulter, Inc. (Fullerton, California) catalog. There are a variety of uses for the dyes and compositions of the invention, which will be readily apparent to one of skill in the art.

In yet another embodiment of the invention, the composition of the invention can contain other dyes and reagents known in the art as blocking agents for specific nucleic acid or proteins within cells, in combination with one or more of the novel dyes of the invention. Such blocking agents are readily selected from among dyes and reagents which have been published and/or which are commercially available. See, for example, Beckman Coulter, Inc. (Fullerton, California) catalog or Molecular Probes (Eugene, Oregon) catalog. There are a variety of uses for the dyes and compositions of the invention, which will be readily apparent to one of skill in the art.

## II. Methods of Staining Nucleic Acids Using the Dyes of the Invention

The dye composition of the invention is useful in staining a variety of nucleic acids, including DNA and RNA.

In samples containing free nucleic acids, for example, nucleic acids not surrounded by an intact cell membrane of a cellular or non-cellular source, the dye composition of the invention can stain the nucleic acids. In one embodiment of the invention, a method is provided for contacting a sample containing nucleic acids with the dye compositions of the invention. Preferably, the method involves contacting a sample containing nucleic acids with a dye composition of the

invention, optionally in the presence of other dyes and reagents known to those of skill in the art. In a preferred embodiment of the invention, the method involves contacting a sample containing nucleic acids with a dye composition of the invention, which contains about 2 to about 10 μM red excitable ReticRed1 dye and an isotonic aqueous buffer. In another preferred embodiment of the invention, the method involves contacting a sample containing nucleic acids with a dye composition of the invention which contains about 1 to about 10 μM of red excitable Dye-1, Dye-2, Dye-3 or combinations thereof and an isotonic aqueous buffer.

The dye composition of the invention are particularly well suited for use in the detection and enumeration of reticulocytes, which contain RNA. The method of the invention therefore involves contacting a reticulocyte, containing nucleic acid, with one or more dye compositions of the invention.

The dye composition of the invention, preferably Groups II and III, are also well suited for use in the detection and enumeration of nucleated red blood cells, which contain DNA.

In one embodiment, the method involves contacting the blood cells with a dye composition of the invention in the presence of at least one surfactant, optionally together with a preservative and sulfonic reagent. As described above, the surfactant(s) can include a detergent and/or a sphering agent. According to the method of the invention, the cells can be contacted with a composition containing these components. Alternatively, one or more of these components can be delivered separately, for example by adding the component(s) directly to the sample. The mixture is then incubated for a suitable period of time. As previously discussed, the incubation time will be less than 1 minute. However, if desired for purposes of convenience, the incubation period may be either extended or shortened. For example, by adjusting the concentration of the dye, surfactant or detergent, the incubation time can be increased to greater than 1 minute. Desirably, this mixing and incubation may be performed at temperatures between approximately 20°C to 40°C. However, in a preferred embodiment of this invention, temperatures ranging from 22°C to 28°C, may be utilized.

In a currently preferred embodiment, the method of the invention requires incubation of the dye for a period ranging from above 0 seconds to about 1 minute to stain reticulocytes, and the staining can be accomplished at room temperatures. In another preferred embodiment, incubation time is eliminated because the blood sample is mixed with the dye composition and thereafter immediately analyzed by the instrument. See Example 5, Fig. 7 and Fig. 8 and Example 7, Figs. 10A-2B, 11A-11C, 12A-12C. Prior art staining techniques using red-excitable dyes for reticulocytes in this time frame require incubation at elevated temperatures and/or additional use of toxic ionophoric compounds.

Reticulocytes stained with the dye compositions, according to the method of the invention, are preferably enumerated in an automatic flow cytometer. However, these cells may also be counted by a manual procedure or automated microscopy.

Thus, the method of the invention facilitates transport of the nucleic acid-specific dyes of this invention across the cell membrane in the time frame desirable for automated flow cytometry. Automatic flow cytometers are well known in the art, and the present invention is not limited to the use of any particular flow cytometer. A preferred embodiment of the invention entails the detection and enumeration of reticulocytes using the XL™ flow cytometer [Beckman Coulter Inc., Miami, Florida]. Different analytical techniques can be employed in the enumeration of reticulocytes by flow cytometric measurements including, but not limited to light scatter, fluorescence, optical absorption, axial light loss, DC electrical impedance, and radio frequency (RF) conductivity. In a preferred embodiment of the invention, in using such flow cytometers, light scatter gates are used to isolate red cells, and fluorescent gates are then used to delineate reticulocytes from mature red cells and enumerate the reticulocytes. In another embodiment of the invention, using flow cytometers, DC and light scatter gates are used to isolate red cells, and fluorescent gates are then used to delineate reticulocytes from mature red cells and enumerate the reticulocytes. In yet another embodiment of the invention, using flow cytometers, DC and fluorescent measurement alone are used to delineate reticulocytes from mature red cells and enumerate the reticulocytes.

Another notable advantage of the preferred embodiment of the invention is that when the composition includes a red-excitable dye of the invention, such as ReticRed1, ReticRed3, Dye-1, Dye-2 or Dye-3, it permits the use of a less expensive excitation source than argon lasers used for other presently available reticulocyte dyes, such as CPO, AO [Seligman, Am. J. Hematology, 14:57 91983)], TO [Lee *et. al.*, Cytometry, 7:508 (1986)], Auramine-O and Pyronin-Y. For example, when the red-excitable dye composition of the invention is used, the present invention advantageously permits the use of a red laser as the excitation source. Specifically, a red diode laser, a high power light emitting diode (LED), or a red helium-neon laser can be used to conduct reticulocyte counting using a red-excitable dye of this invention.

A variety of lasers are known to those of skill in the art that may be utilized as the excitation sources for the flow cytometers. The lasers may be selected from, but are not limited to, argon, helium-neon, diode, and diode pumped solid-state lasers, depending upon the excitation wavelength of the dye of the invention selected for detection of reticulocytes. Selection of suitable light sources, and appropriate excitation wavelengths, are not a limitation on the present invention.

Suitable excitation wavelengths may be readily determined by one of skill in the art. Examples of suitable excitation wavelengths in the red spectral range include those in the range of 600 nm to 725 nm, and preferably 630 nm to 670 nm. Other suitable excitation sources and wavelengths may be readily selected by one of skill in the art, taking into consideration the dye selected for use in the method and compositions of the invention.

An additional advantage comes from the fact that red-excitable dye compounds of this invention such as ReticRed1, ReticRed2, ReticRed3, ReticRed4, ReticRed5, ReticRed6, Dye-1, Dye-2 or Dye-3 etc., are essentially non-fluorescent or very weakly fluorescent in aqueous solutions in the absence of nucleic acids (unbound state). As a result, problems associated with background fluorescence are minimal, and reticulocytes can be detected with high sensitivity using this dye.

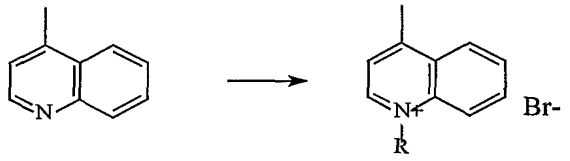
Thus, the present invention permits reticulocytes to be rapidly stained with the dyes of the invention for subsequent flow analysis. The method of the invention differs from both the prior art Pyronin-Y [Tanke et al, cited above] staining procedure requiring fixation of the cells and the prior art fluorescence staining procedures involving membrane permeable dyes such as CPO (reticONE™) or Thiazole Orange (Retic-Count™) that does not require cell-fixation, but requires long incubation with the blood to accomplish staining.

## III. Examples

The following examples are provided to illustrate the invention and do not limit the scope thereof. One skilled in the art will appreciate that although specific reagents and conditions are outlined in the following examples, modifications can be made which are meant to be encompassed by the spirit and scope of the invention.

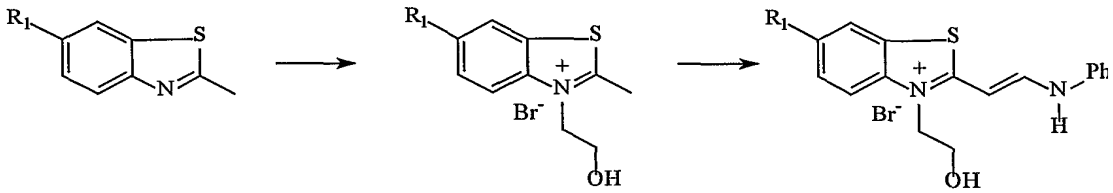
Example 1A - Materials and Methods for Synthesis of Group I Compounds

A. *General Dye Synthesis for Group I*



1

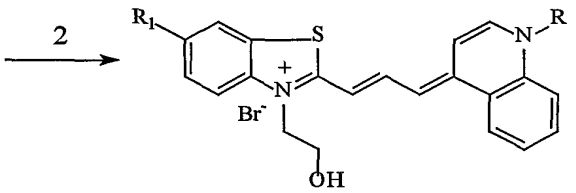
2, R = $CH_2$ $CH_2OH$



3a, $R_1$ = H
3b, $R_1$ = $CH_3$
3c, $R_1$ = $OCH_3$

4a, $R_1$ = H
4b, $R_1$ = $CH_3$
4c, $R_1$ = $OCH_3$

5a, $R_1$ = H
5b, $R_1$ = $CH_3$
5c, $R_1$ = $OCH_3$



6, R = $CH_2CH_2OH$, $R_1$ = H

7, R = $CH_2CH_2OH$, $R_1$ = $CH_3$

5

*B. Specific Dye Preparation*

**Preparation of 1-(2-Hydroxy)ethyllepidinium Bromide (2).** A solution of lepidine (11.5 g) and bromoethanol (100 g) were stirred and heated in an oil bath at 110°C for 48 hours. Cooling the reaction mixture to room
10 temperature, adding ethyl acetate (100 ml) and stirring for 5 min resulted in a solid that was collected, washed with ethyl acetate (2 x 20 ml) and dried. The solid was then dissolved in methanol (80 ml) and precipitated with ethyl acetate (600 ml). The solid was collected via filtration, washed with ethyl acetate (2 x 100 ml) and

dried in an oven under high vacuum at 50°C overnight to obtain 11.47 g (53% yield) of product (2c).

**Preparation of 3-(2-Hydroxy)ethyl-2-methylbenzothiazolium Bromide (4a).** A solution of 2-methylbenzothiazole (5.87 g) and 2-bromoethanol (49.2 g) was stirred and heated in an oil bath at 110°C for 18 hours. After cooling the reaction mixture to room temperature, adding ethyl acetate (100 ml), and decanting, the resultant solid was then dissolved in methanol (50 ml) and precipitated with ethyl acetate (300 ml). The solid was again recrystallized from a mixture of methanol and ethyl acetate, washed with ethyl acetate (2 x 25 ml) and dried in an oven under high vacuum at 50°C overnight to obtain 4.21 g (39%) of 4a.

**Preparation of 3-(2-Hydroxy)ethyl-2,6-dimethylbenzothiazolium Bromide (4b).** A solution of 2,6-dimethylbenzothiazole (13.0 g) and 2-bromoethanol (100 g) was stirred and heated in an oil bath to 120°C for 96 hours. After cooling the reaction mixture to room temperature, adding ethyl acetate (150 ml) and stirring for 1 hour, the resultant solid was filtered and collected. The solid was then dissolved in methanol (150 ml) and charcoal (2g) was added. The charcoal was removed by passing through a pad of Celite®. The solution was then concentrated to a small volume and triturated with acetone (200 ml). The solid was collected, washed with acetone (2 x 30 ml), ethyl acetate (2 x 30 ml) and dried in an oven under high vacuum at 50 C overnight to obtain 13.95 g (61%) of 4b.

**Preparation of 3-(2-Hydroxy)ethyl-6-methoxy-2-methylbenzothiazolium Bromide (4c).** A solution of 6-methoxy-2-methylbenzothiazole (1.82 g) and 2-bromoethanol (8.8 g) was stirred and heated in an oil bath at 120°C for 74 hours. After cooling the reaction mixture to room temperature, adding ethyl acetate (25 ml) and stirring for 1 hour, the solid was filtered and collected. The solid was then dissolved in methanol (50 ml) and charcoal (0.5g) was added. The charcoal was removed by passing through a pad of Celite®. The solution was then concentrated to a small volume and triturated with ethyl acetate (100 ml). The solid was collected, washed with ethyl acetate (2 x 30

ml) and dried in an oven under high vacuum at 50°C overnight to obtain 0.9 g (30%) of 4c.

**Preparation of 3-(2-Hydroxy)ethyl-2-(2-N-phenyl)ethenylbenzothiazolium Bromide (5a).** To a solution of 3-(2-hydroxy)ethyl-2-methylbenzothiazolium bromide (**4a**, 1.19 g) in a mixed solvent of methanol/ethanol (3:1, 80 ml) was added an excess of ethyl N-phenylformimidate (3.0 g) and the solution was stirred at room temperature for 36 h. The solvent was removed under reduced pressure to give a dry solid. The solid was subjected to column chromatography (silica gel, methylene chloride/methanol) purification. The fractions containing the product were combined and concentrated to dryness under reduced pressure. The solid was dissolved in methanol (10 ml) and precipitated with ethyl ether (200 ml). After drying at 50°C under high vacuum overnight, 1.04 g (63%) of 5a was obtained as a yellow solid.

**Preparation of 3-(2-Hydroxy)ethyl-6-methyl-2-(2-N-phenyl)ethenylbenzothiazolium Bromide (5b).** To a solution of 3-(2-hydroxy)ethyl-2,6-dimethylbenzo-thiazolium bromide (**4b**, 2.76 g) in methanol (80 ml) was added an excess of ethyl N-phenylformimidate (3.6 g) and the solution stirred at room temperature for 36 hours. Ethyl acetate (160 ml) was then added and stirred overnight. The solid was collected, washed with ethyl acetate (2 X 30 ml) and dried. Drying the solid further in an oven at 50°C under high vacuum overnight afforded 2.32 g (62%) of 5b as a yellow solid.

**Preparation of 3-(2-Hydroxy)ethyl-6-methoxy-2-(2-N-phenyl)ethenylbenzothiazolium Bromide (5c).** To a solution of 3-(2-hydroxy)ethyl-6-methoxy-2-methylbenzothiazolium bromide (**4c**, 0.79 g) in methanol (30 ml) was added an excess of ethyl N-phenylformimidate (1.5 g) and the solution was stirred at room temperature for 16 h. The solvent was removed under reduced pressure to yield a dry solid. The solid was subjected to column chromatography (silica gel), and eluted with a methanol/methylene chloride gradient (from 0 to 15% methanol). The fractions containing the product were combined and concentrated to dryness under reduced pressure. The solid was dissolved in methanol (5 ml) and precipitated with ethyl ether (150 ml). After

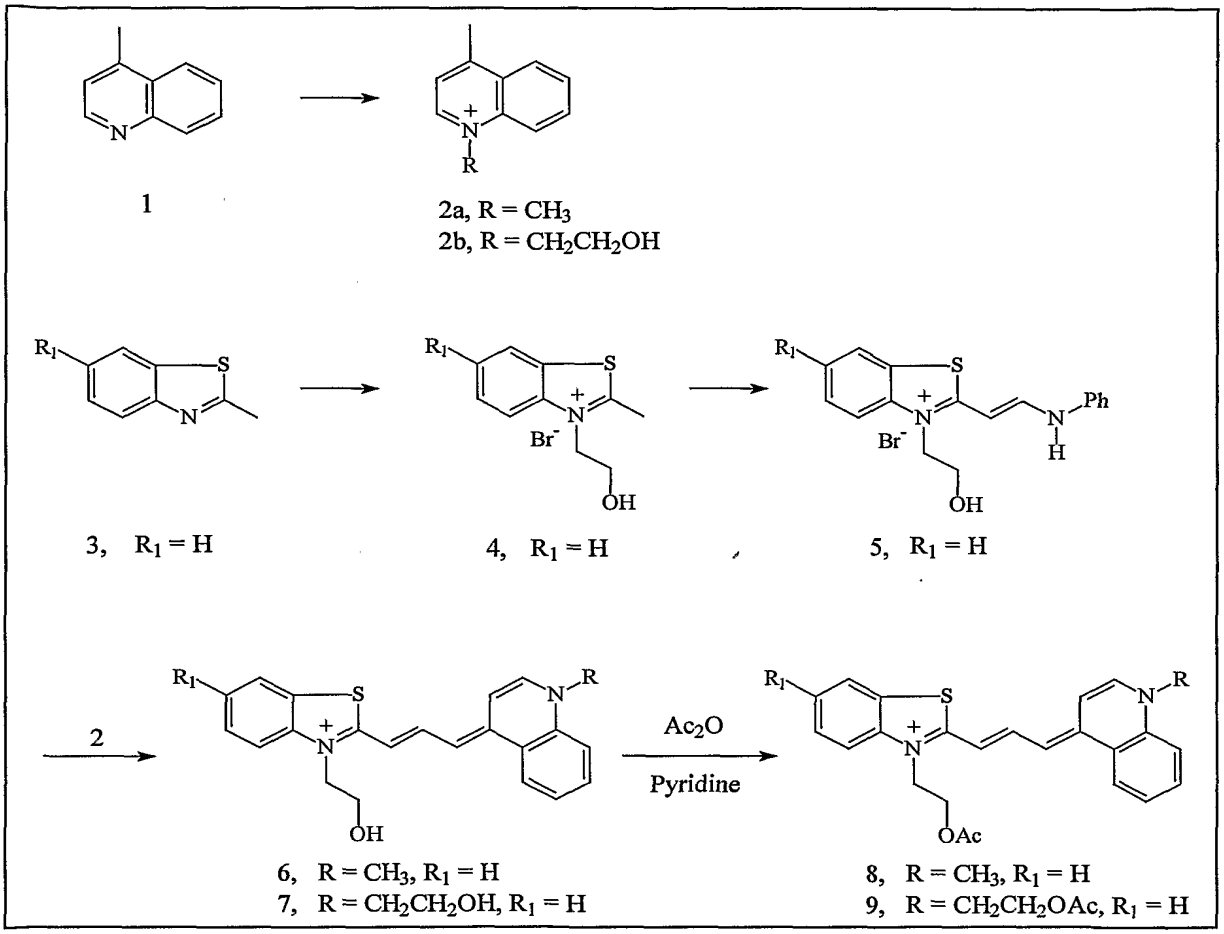
drying in an oven at 50°C under high vacuum overnight, 0.35 g (33%) of 5c as a yellow solid was obtained.

**Preparation of Dye Compound 6 (Retic Red1).** To a solution of 3-(2-hydroxy)ethyl-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**5a**, 85.8 mg) and 1-(2-hydroxy)ethyllepidinium bromide (**2**, 58.4 mg) in methylene chloride (20 ml) was added triethylamine (91 μL) and acetic anhydride (62 μL). After stirring at room temperature for 2 hours the solvent was removed under reduced pressure to give a blue solid. The solid was partially redissolved in methanol (15 ml) and ethyl acetate (300 ml) was added. The resultant solid was collected via filtration, washed with ethyl acetate (2 X 30 ml) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 67.8 mg (66%) of 8 as a blue solid. Absorption spectrum maximum: 630 nm (in methanol), 640 nm (in DMSO).

**Preparation of Dye Compound 7 (ReticRed6).** To a solution of 3-(2-hydroxy)ethyl-6-methyl-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**5b**, 85.6 mg), 1-(2-hydroxy)ethyllepidinium bromide (**2**, 58.7 mg) in methylene chloride (10 ml), and methanol (1.0 ml) was added triethylamine (91 μL) and acetic anhydride (61 μL). After stirring at room temperature for 2 hours the solvent was removed under reduced pressure to give a blue solid. The solid was partially redissolved in methanol (10 ml) and ethyl acetate (200 ml) was added. The resultant solid was collected via filtration, washed with ethyl acetate (2 x 30 ml) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 63.7 mg (60%) of 11 as a blue solid. Absorption spectrum maximum: 636 nm (in methanol), 645 nm (in DMSO).

Example 1B : Materials and Methods for Synthesis of Group-II Compounds

*Synthetic Scheme*




**(i) Preparation of 1-Methyllepidinium Iodide (2a).** A solution of lepidine (10.56 g) and methyliodide (105 g) was stirred and heated to reflux in an oil bath at 50°C for 19 hours. Cooled the reaction mixture to room temperature, added acetone (200 mL) and stirred for 2 hours. The resultant solid was collected, washed with ethyl acetate (2 x 20 mL) and then triturated with ethyl acetate (200 mL). The solid was collected via filtration, washed with ethyl acetate (2 x 25 mL)

and dried in an oven under high vacuum at 50°C overnight. There was obtained 20.74 g (98.6% yield) of product.

**(ii) Preparation of 1-(2-Hydroxyethyl)lepidinium Bromide (2b).** A solution of lepidine (11.5 g) and bromoethanol (100 g) was stirred in an oil bath at 110°C for 48 hours. Cooled the reaction mixture to room temperature, added ethyl acetate (100 mL) and stirred for 5 min. The resultant solid was collected, washed with ethyl acetate (2 x 20 mL) and dried. The solid was then dissolved in methanol (80 mL) and precipitated with ethyl acetate (600 mL). The solid was collected via filtration, washed with ethyl acetate (2 x 100 mL) and dried in an oven under high vacuum at 50°C overnight. There was obtained 11.47 g (53% yield) of product.

**(iii) Preparation of 3-(2-Hydroxyethyl)-2-methylbenzothiazolium Bromide (4).** A solution of 5.87 g of 2-methylbenzothiazole (**3**) and bromoethanol (49.2 g) was stirred in an oil bath at 110°C for 18 hours. Added ethyl acetate (100 mL) to the reaction mixture and decanted. The residual solid was then dissolved in methanol (50 mL) and precipitated with ethyl acetate (300 mL). The solid was again recrystallized from methanol-ethyl acetate (15 mL/150 mL), washed with ethyl acetate (2 x 25 mL) and dried in an oven under high vacuum at 50°C overnight. The yield was 4.21 g (39%). TLC (silica gel, 4:1 methylene chloride:methanol) $R_f$=0.34.

**(iv) Preparation of 3-(2-Hydroxyethyl)-2-(2-N-phenyl) ethenylbenzothiazolium Bromide (5).** To a solution of 3-(2-hydroxyethyl)-2-methylbenzothiazolium bromide (**4**, 1.19 g) in a mixed solvent of methanol/ethanol (3:1, 80 mL) was added excess of ethyl N-phenylformidiate (3.0 g) and stirred at room temperature for 36 hours. Evaporated off the solvent under reduced pressure to dryness. The solid was subjected to column chromatography (silica gel, methylene chloride/methanol) purification. The fractions contained the product were combined and concentrated to dryness under reduced pressure. The solid was dissolved in methanol (10 mL) and precipitated with ethyl ether (200 mL). After drying in an oven at 50°C under high vacuum overnight there was obtained 1.04 g

(63%) of a yellow solid. TLC (silica gel, 9:1 methylene chloride:methanol) $R_f$= 0.27.

**(v) Preparation of dye compound corresponding to the configuration described as 6 in the above figures, having R=$CH_3$, R1=H.** A solution of 3-(2-hydroxyethyl)-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**5**, 203.8 mg) and 1-methyllepidinium iodide (**2a**, 154.0 mg) in pyridine (10 mL) was heated in an oil bath at 90°C for 2 hours under anhydrous condition. A solution of sodium tetrafluoroborate (59.3 mg) in DMF (0.5 mL) was added and the heating continued for another 20 min at 90°C. After cooling to room temperature the reaction solution was poured into ethyl acetate (100 mL) and the resultant solid was collected via filtration. Re-dissolved the solid in methanol (100 mL) and added ethyl acetate (300 mL) and stirred overnight. The resultant solid was collected via filtration, washed with ethyl acetate (2 x 20 mL) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 165.4 mg (68%) of a blue solid. TLC (silica gel, 4:1 methylene chloride:methanol) $R_f$= 0.65. Maximum Absorption: 629 nm (in methanol).

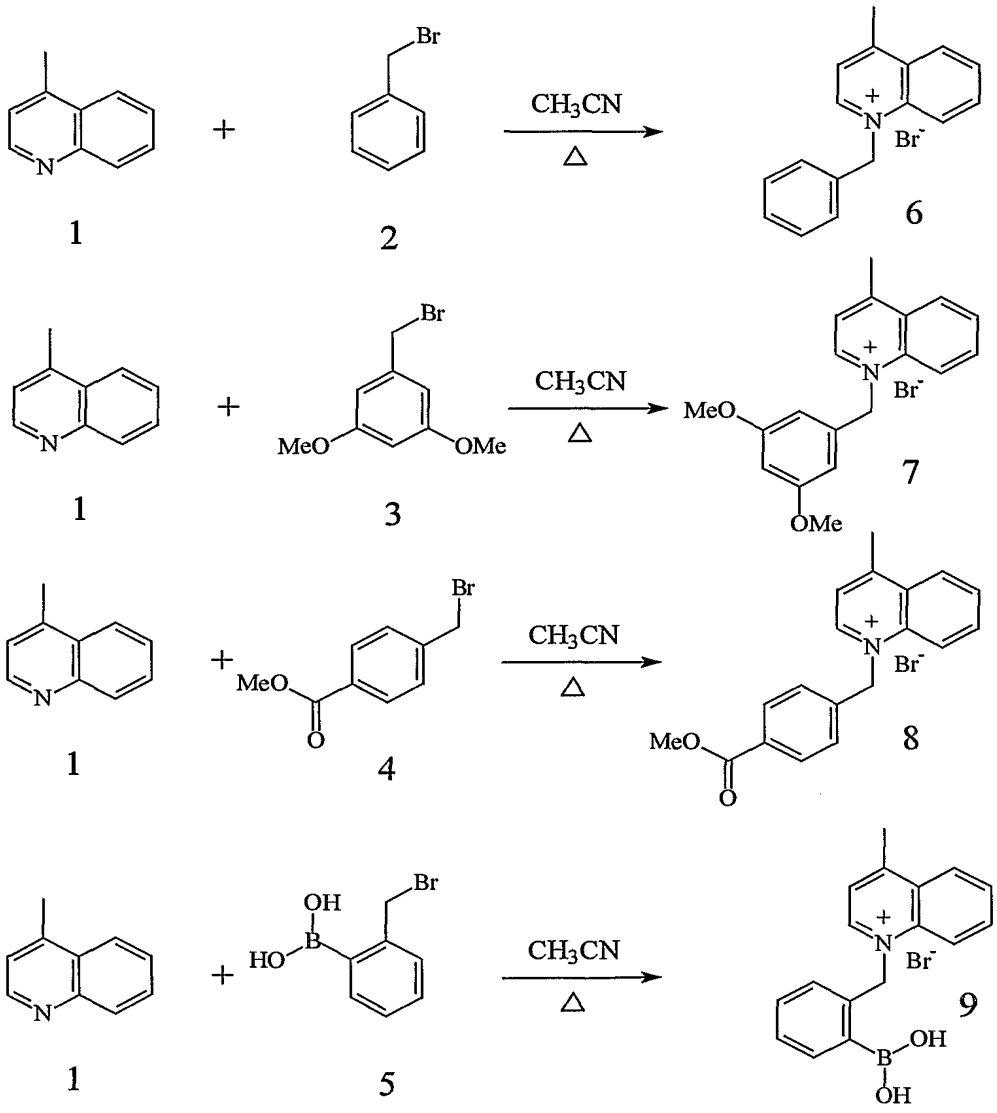
**(vi) Preparation of the dye compound corresponding to the configuration described as 7, having R=$CH_2CH_2$(OH), R1=H.** To a solution of 3-(2-hydroxyethyl)-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**5**, 85.8 mg) and 1-(2-hydroxyethyl)lepidinium bromide (**2b**, 58.4 mg) in methylene chloride (20 mL) was added triethylamine (91 μL) and acetic anhydride (62 μL). After stirring at room temperature for 2 hours the solvent was evaporated off under reduced pressure to give a blue solid. Partially redissolved the solid in methanol (15 mL) and added ethyl acetate (300 mL). The resultant solid was collected via filtration, washed with ethyl acetate (2 x 30 mL) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 67.8 mg (66%) of a blue solid. TLC (silica gel, 4:1 methylene chloride:methanol) $R_f$= 0.18. Maximum Absorption: 631 nm (in methanol).

**(vii) Preparation of the acetylated dye compound corresponding to the molecular configuration described as 8.** A suspension of dye compound described in (v) above (50 mg) in acetic anhydride (4 mL) and pyridine (1 mL) was heated at 50°C with stirring in an oil bath under anhydrous condition for 10 hours. After cooling to room temperature the reaction solution was poured into ethyl acetate (100 mL) and stirred for 3 min. The resultant fine crystals were collected via filtration, washed with ethyl acetate (2 x 20 mL) and dried in an oven at 50°C under high vacuum overnight. There was obtained 56.5 mg (quantitative yield) of a blue crystalline solid product. TLC (silica gel, 4:1 methylene chloride:methanol) $R_f$= 0.79 Maximum Absorption: 625 nm (in methanol). This compound is referenced hereinafter as Dye-1.

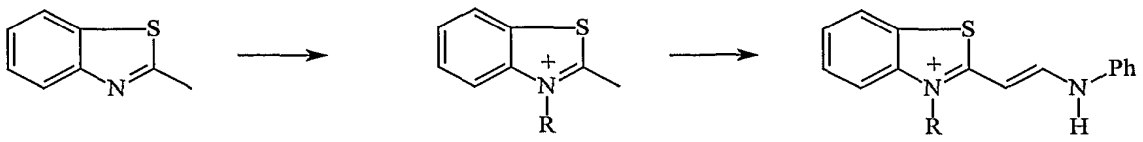
**(viii) Preparation of the acetylated dye compound corresponding to the molecular configuration described as 9.** A suspension of dye compound described in (vi) above, (377.5 mg) in acetic anhydride (8 mL) and pyridine (2 mL) was heated at 50°C with stirring in an oil bath under anhydrous condition for 10 hours. After cooling to room temperature the reaction solution was poured into ethyl acetate (100 mL) and stirred for 3 min. The resultant solid was collected via filtration, washed with ethyl acetate (2 x 20 mL), ethyl ether (2 x 20 mL) and dried in an oven at 50°C under high vacuum overnight. There was obtained 411.9 mg (92.6% yield) of a blue solid product. TLC (silica gel, 4:1 methylene chloride:methanol) $R_f$= 0.96. Maximum Absorption: 631 nm (in methanol). This compound is referenced hereinafter as Dye-2.

Example 1C - Materials and Methods for Synthesis of Group III Compounds

*Synthetic Scheme I.*



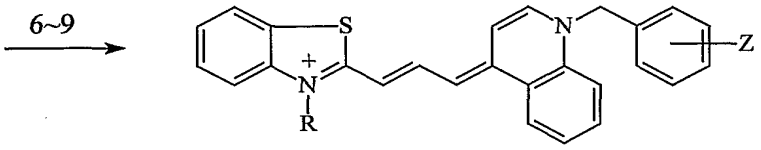
*Synthetic Scheme II.*



10

11a, R = $CH_3$
11b, R = $CH_2CH_2OH$

12a, R = $CH_3$
12b, R = $CH_2CH_2OH$



13, R = $CH_2CH_2OH$, Z = H
14, R = $CH_2CH_2OH$, Z = 3'-5'-$(OMe)_2$
15, R = $CH_2CH_2OH$, Z = 4'-$COOCH_3$
16, R = $CH_3$, Z = 2'-$B(OH)_2$

**Preparation of 2-Bromomethylphenylboronic Acid (5).** A suspension of 2-methylphenylboronic acid (10.65 g), N-bromosuccinimide (NBS, 16.86 g) and 2,2'-azobisisobutyronitrile (AIBN, 1.708 g) in carbon tetrachloride (1000 mL) was heated in an oil bath at 82°C to reflux for 2 hours under anhydrous condition. Cooled the reaction mixture to room temperature and filtered off the solid. Extracted the filtrate with water (2 x 200 mL). The organic layer was separated, dried over $MgSO_4$ and concentrated in vacuo to dryness. Redissolved the solid in methanol (100 mL) and added ethyl acetate (1000 mL) to it. The resultant precipitate was collected, washed with ethyl acetate (2 x 50 mL) and dried in the air. The filtrate was concentrated to a small volume and a second crop of solid was also collected. The combined solid was dried in an oven under high vacuum at 50°C overnight. There was obtained 6.48 g (39%) of product.

**Preparation of 1-Benzyllepidinium Bromide (6).** A solution of lepidine (37.82 g) and benzyl bromide (6.471 g) in acetonitrile (100 mL) were stirred and heated to reflux in an oil bath at 93°C for 20 hours. Cooled the reaction mixture to room temperature, added ethyl acetate (50 mL) and stirred for 30 min. The resultant solid was collected, washed with ethyl acetate (4 X 20 mL) and dried in the air. The filtrate was concentrated to a small volume and a second crop of solid was also collected. The combined solid was dried in an oven under high vacuum at 50°C overnight. There was obtained 11.52 g (96.9%) of product.

**Preparation of 1-(3'-5'-Dimethoxybenzyl)lepidinium Bromide (7).** A solution of lepidine (3.36 g) and 3,5-dimethoxybenzyl bromide (5.41 g) in acetonitrile (100 mL) were stirred and heated to reflux in an oil bath at 90°C for 30 hours. Cooled the reaction mixture to room temperature, added ethyl acetate (50 mL) and stirred for 30 min. The resultant solid was collected, washed with ethyl acetate (4 X 20 mL) and dried in the air. Redissolved the solid in methanol (200 mL) and treated with charcoal (5 g) and heated to reflux for 15 min to remove the dark color impurities. After filtering through a pad of Celite® the filtrate was concentrated to a small volume (~20 mL) and ethyl acetate (300 mL) was added. The resultant precipitate was collected, washed with ethyl acetate (2 X 50 mL), ethyl ether (2 X 20 mL) and dried in the air. Further drying in an oven under high vacuum at 50°C overnight provided 6.65 g (76%) of product.

**Preparation of 1-(4'-Carboxymethylbenzyl)lepidinium Bromide (8).** A solution of lepidine (3.32 g) and methyl 4-(bromomethyl)benzoate (5.31 g) in acetonitrile (100 mL) were stirred and heated to reflux in an oil bath at 90°C for 16 hours. After cooling to room temperature the solvent was evaporated off under reduced pressure to dryness. The resultant solid was washed with ethyl acetate (3 X 100 mL). Redissolved the solid in methanol (100 mL) and treated with charcoal (5 g) and heated to reflux for 15 min to remove the dark color impurities. After filtering through a pad of Celite® the filtrate was concentrated to dryness. The solid was then triturated with acetone (100 mL). The resultant solid was collected, washed with ethyl acetate (2 X 50 mL) and dried in the air. The filtrate was

concentrated to a small volume and a second crop of solid was also collected. The combined solid was dried in an oven under high vacuum at 50°C overnight. There was obtained 7.26 g (84%) of product.

**Preparation of 1-(2'-Boronic acid)benzyllepidinium Bromide (9).** A solution of lepidine (306 mg) and 2-bromomethylphenylboronic acid (417 mg) in acetonitrile (12 mL) were stirred and heated to reflux in an oil bath at 90°C for 17 hours. After cooling to room temperature the solvent was evaporated off under reduced pressure to dryness. Washed the solid with ethyl acetate (3 X 20 mL) and the solvent was decanted. Redissolved the solid in methanol (3 mL) and again concentrated to dryness. Washed the solid with ethyl acetate (3 X 20 mL). The solid was then dried in an oven under high vacuum at 50°C overnight. There was obtained 650 mg (93.5%) of product.

**Preparation of 2,3-Dimethylbenzothiazolium Iodide (11a).** A solution of 2-methylbenzothiazole (5.00 g) and iodomethane (50 g) were stirred in an oil bath at 45°C for 44 hours. After cooling to room temperature the solid was collected through filtration and the solid was washed with ethyl acetate (2 X 100 mL) and dried in an oven under high vacuum at 50°C overnight. There was obtained 8.02 g (82.3% yield) of product.

**Preparation of 3-(2-Hydroxy)ethyl-2-methylbenzothiazolium Bromide (11b).** A solution of 2-methylbenzothiazole (5.87 g) and bromoethanol (49.2 g) were stirred in an oil bath at 110°C for 18 hours. Added ethyl acetate (100 mL) to the reaction mixture and decanted. The residual solid was then dissolved in methanol (50 mL) and precipitated with ethyl acetate (300 mL). The solid was again recrystallized from methanol-ethyl acetate (15 mL/150 mL), washed with ethyl acetate (2 X 25 mL) and dried in an oven under high vacuum at 50°C overnight. The yield was 4.21 g (39%). TLC (silica gel, 4:1 methylene chloride-methanol) $R_f$=0.34.

**Preparation of 3-Methyl-2-(2-N-phenyl)ethenylbenzothiazolium Iodide (12a).** To a solution of 2,3-dimethylbenzothiazolium bromide (**11a**, 1.01 g) in ethanol (30 mL) was added excess of ethyl N-phenylformidiate (1.5 g) and stirred at room temperature for 4 days. Evaporated off solvent under reduced pressure to dryness. The solid was subjected to column chromatography (silica gel, methylene chloride/methanol) purification. The fractions contained the product were combined and concentrated to dryness under reduced pressure. The solid was dissolved in methanol (10 mL) and precipitated with ethyl ether (200 mL). After drying in an oven at 50°C under high vacuum overnight there was obtained 0.88 g (60%) of a yellow solid. TLC (silica gel, 4:1 methylene chloride-methanol) $R_f$=0.60.

**Preparation of 3-(2-Hydroxy)ethyl-2-(2-N-phenyl)ethenylbenzothiazolium Bromide (12b).** To a solution of 3-(2-hydroxy)ethyl-2-methylbenzothiazolium bromide (**11b**, 1.19 g) in a mixed solvent of methanol/ethanol (3:1, 80 mL) was added excess of ethyl N-phenylformidiate (3.0 g) and stirred at room temperature for 36 h. Evaporated off solvent under reduced pressure to dryness. The solid was subjected to column chromatography (silica gel, methylene chloride/methanol) purification. The fractions contained the product were combined and concentrated to dryness under reduced pressure. The solid was dissolved in methanol (10 mL) and precipitated with ethyl ether (200 mL). After drying in an oven at 50°C under high vacuum overnight there was obtained 1.04 g (63%) of a yellow solid. TLC (silica gel, 4:1 methylene chloride-methanol) $R_f$=0.58.

**Preparation of Dye Compound designated as 13 under *Synthesis Scheme II* in Example 1C:**

To a solution of 3-(2-hydroxy)ethyl-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**12b**, 41.9 mg) and 1-benzyllepidinium bromide (**6**, 33.4 mg) in methylene chloride (5 mL) was added triethylamine (44.3 μL) and acetic anhydride (30.1 μL). After stirring at room temperature for 1 hour the solvent was evaporated off under reduced pressure to give a blue solid.

Redissolved the solid in methanol (10 mL) and added ethyl acetate (200 mL). The resultant solid was collected via filtration, washed with ethyl acetate (3 X 10 mL) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 49.1 mg (89%) of a blue solid. TLC (silica gel, 9:1 methylene chloride-methanol) $R_f$=0.43. Absorption Spectrum: 636 nm (methanol).

**Preparation of Dye Compound designated as 14 under *Synthesis Scheme II* in Example 1C:**

To a solution of 3-(2-hydroxy)ethyl-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**12b**, 120.5 mg) and 1-(3'-5'-dimethoxybenzyl) lepidinium bromide (**7**, 119.5 mg) in methylene chloride (15 mL) and methanol (1 mL) was added triethylamine (133 μL) and acetic anhydride (90 μL). After stirring at room temperature for 30 min the solvent was evaporated off under reduced pressure to give a blue solid. Redissolved the solid in methanol (10 mL) and added acetone (100 mL) and ethyl acetate (100 mL). The resultant solid was collected via filtration, washed with ethyl acetate (3 X 20 mL) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 147.3 mg (80%) of a blue solid. TLC (silica gel, 4:1 methylene chloride-methanol) $R_f$=0.91. Absorption Spectrum: 637 nm (methanol).

**Preparation of Dye Compound designated as 15 under *Synthesis Scheme II* in Example 1C:**

To a solution of 3-(2-hydroxy)ethyl-2-(2-N-phenyl)ethenylbenzothiazolium bromide (**12b**, 171.1 mg) and 1-(4'-carboxymethyl-benzyl)lepidinium bromide (**8**, 168.8 mg) in methylene chloride (15 mL) and methanol (1 mL) was added triethylamine (189 μL) and acetic anhydride (128 μL). After stirring at room temperature for 1 h the solvent was evaporated off under reduced pressure to give a blue solid. Redissolved the solid in methanol (10 mL) and added acetone (50 mL) and ethyl acetate (50 mL). The resultant solid was collected via filtration, washed with ethyl acetate (3 X 30 mL) and dried. Further drying the solid in an oven at 50°C under high vacuum

overnight afforded 208.9 mg (80%) of a blue solid. TLC (silica gel, 4:1 methylene chloride-methanol) $R_f$=0.80. Absorption Spectrum: 639 nm (methanol).

**Preparation of Dye Compound designated as 16 under** *Synthesis Scheme II* **in Example 1C:**

To a solution of 3-methyl-2-(2-N-phenyl)ethenylbenzothiazolium iodide (**12a**, 144.6 mg) and 1-(2'-boronic acid)benzyllepidinium Bromide (**9**, 131.3 mg) in methylene chloride (5 mL) and methanol (1 mL) was added triethylamine (153 μL) and acetic anhydride (104 μL). After stirring at room temperature for 15 min the solvent was evaporated off under reduced pressure to give a blue solid. Redissolved the solid in methanol (5 mL) and added ethyl acetate (200 mL). The resultant solid was collected via filtration, washed with ethyl acetate (3 X 30 mL) and dried. Further drying the solid in an oven at 50°C under high vacuum overnight afforded 107 mg (50%) of a blue solid. TLC (silica gel, 4:1 methylene chloride-methanol) $R_f$= 0.58. Absorption Spectrum: 634 nm (methanol).

*D. Dye Stock Solution*

Stock solutions of dyes at 5 mM concentration in dimethylsulfoxide (DMSO) were prepared by dissolving a definite quantity of each dye compound in appropriate volume of DMSO.

*E. Dye Spectroscopy*

For spectroscopic measurements, individual sample solution of each dye in PBS was prepared by diluting portions of its stock solution into phosphate-buffered saline (PBS). Specifically, in one experiment, the dye solutions were prepared by mixing 2 μl of the 5mM stock dye solution with 5 ml of PBS to give a final 2 μM dye concentration. Fluorescence spectra of the above solutions were measured with a spectrofluorometer using an excitation wavelength of 633 nm.

Solutions of the dyes of this invention bound to RNA were prepared by combining 2 μl of the 5mM stock solution of the dye and a 5 ml solution of RNA dissolved in PBS at a RNA concentration of 1 mg/ml. Fluorescence spectrum of

each Dye/RNA solution was measured with a spectrofluorometer using an excitation wavelength of 633 nm.

Solutions of the dyes of this invention bound to DNA were prepared by combining 2 µl of the 5mM stock solution of the dye and a 5 ml solution of DNA dissolved in PBS at a DNA concentration of 0.5 mg/ml Fluorescence spectrum of each Dye/DNA solution was measured with a spectrofluorometer using an excitation wavelength of 633 nm.

*F. Flow Cytometry For Red Excitable Dyes*

An XL™ flow cytometer [Beckman Coulter Inc., Miami, Florida] was used to conduct experiments described herein using the red-excitable dyes of the invention. The flow cytometer was modified from a standard XL™ flow cytometer by incorporating a HeNe laser (632.8 nm) and two red-sensitive photomultiplier tubes (PMT). Approximately 11.5 mW of 632.8 nm laser light from the above laser was incident on the beam shaping optics of the flow cytometer. Forward light scatter (FS), side scatter (SS) and one fluorescence (FL) parameter in the orthogonal direction were measured to analyze the red cells. The red cells were gated on a SS vs. FS dotplot. A fixed gate was then used to enumerate retics on a FL vs. FS dotplot.

*G. Reference Analyses*

Separate analyses for reticulocytes were performed using either (1) the standard Beckman Coulter reticulocyte reagents reticONE™, which is based on a blue-excitable metachromatic fluorescent dye compound, employing a standard XL™ flow cytometer [Beckman Coulter Inc., Miami, Florida] having a 488 nm argon laser as the illumination source, or (2) the conventional Retic-Count™ reticulocyte enumeration method (Becton Dickinson Cat. #349204) using Thiazole orange excited at 488 nm in a FACSCAN™ flow cytometer. In the reticONE™ method, FS, SS, and two FL parameters were measured to analyze the red cells stained with the blue-excitable fluorescent dye contained in the reticONE™ reagent. Out of the two FL parameters measured, one is measured at 525 nm,

which corresponds to fluorescence due to the dye bound to DNA, and the other is measured at 675 nm, which corresponds to the dye bound to RNA. An automated gating algorithm, available in commercial XL™ flow cytometers, calculated reticulocyte percentage from a DNA vs. RNA fluorescence dotplot. This method is described in U.S. Patent No. 5639,666.

For the Retic-Count™ method, FS, SS, and one FL parameter were measured to analyze the red cells stained with the blue-excitable fluorescent dye Thiazole Orange contained in the Retic-Count™ reagent.

Example 2 - Fluorescence Comparison of the Dyes of present invention in Bound and Unbound States

In order to identify a fluorescent dye as a suitable candidate for reticulocyte enumeration, the fluorescence properties of several different compounds were determined. For reticulocyte enumeration, it is most desirable to have dyes that are weakly fluorescent when they are not bound to RNA, but show significant enhancement in fluorescence intensity when they are bound to RNA. However, it is not possible to predict which dye compound will have this ideal combination effects on its fluorescence activity both in absence and in presence of RNA. Significantly, the inventors found that the dyes of the present invention had favorable fluorescence properties required for sensitive detection of RNA.

For fluorescence measurements, a stock solution of each compound in DMSO was made (as described in Example 1D). A portion of this stock solution was diluted in PBS to obtain a dye concentration of 2 μM. 1 ml of this dilute solution was placed in a glass cuvette and the fluorescence spectra obtained exciting the dye at its excitation wavelength maximum. Next, 1ml of a solution of free calf liver RNA (Sigma) dissolved in PBS at a concentration of 8 mg/ml, and a measured volume of the dye stock solution was mixed to obtain a dye concentration of 2 μM in the RNA solution. This dye solution diluted in the RNA solution was placed in a glass cuvette and the fluorescence spectra measured using the same excitation as in the previous measurement. As an example, Fig. 1A shows the comparison of the fluorescence spectra of the dye compound ReticRed1 in PBS

solution with (see the upper curve) and without RNA (see the lower curve) present in the solution.

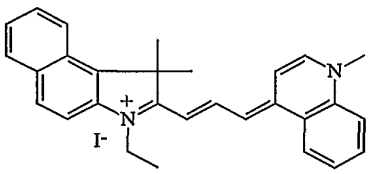
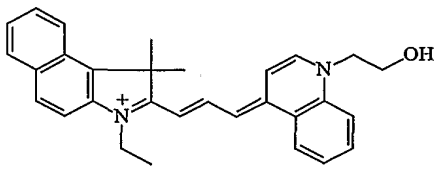
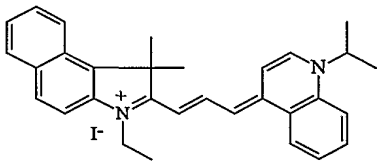
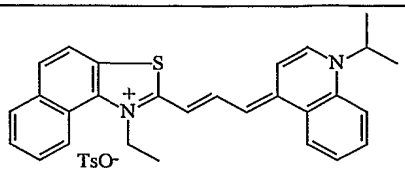
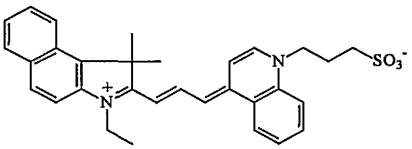
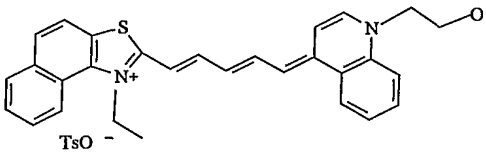
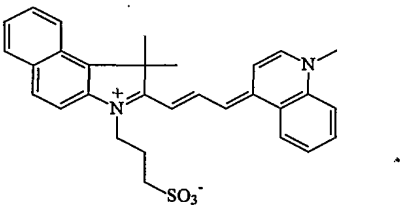
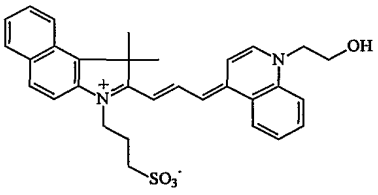
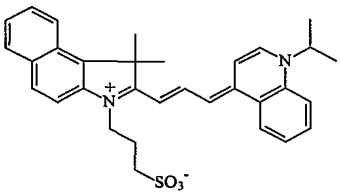
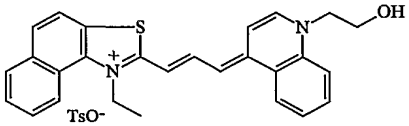
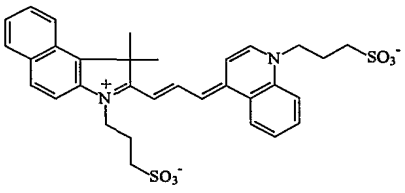
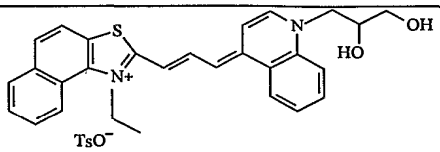
In Fig. 1B, comparison of fluorescence intensity of several compounds in presence of RNA in solution are presented. In this Fig. 1B, the fluorescence intensities of the dyes are shown relative to the fluorescence intensity of a commercial dye Syto 62 (Molecular Probes Inc.) used herein as a reference standard. The structures of the additional compounds included in this study and shown in Fig. 1B but not already described above are shown in Table 2.

Table 1 compares the fluorescence intensities of the dyes of the present invention, ReticRed1 through ReticRed6 (concentration 2 μM), when they are not bound to RNA (unbound to RNA) and when they are in presence of RNA (bound to RNA). These intensities were obtained at the peak emission wavelengths of each fluorescence spectra measured as described above. Fig. 1C compares the fluorescence intensities of the RNA-bound and unbound dyes in graphical form.

**Table-1**

| DYE | Fluorescence Intensity | | Fluorescence enhancement ratio (Bound/Unbound) |
|---|---|---|---|
| | Unbound to RNA | Bound to RNA | |
| ReticRed1 | 2.49 | 791.23 | 317.763 |
| ReticRed2 | 1.72 | 643.77 | 374.285 |
| ReticRed3 | 1.28 | 639.68 | 499.75 |
| ReticRed4 | 1.72 | 713.4 | 414.767 |
| ReticRed5 | 2.24 | 656.06 | 292.884 |
| ReticRed6 | 2.75 | 619.2 | 225.164 |

**TABLE 2**

| | | | |
|---|---|---|---|
| GS6262-43 |  | GS6262-82 |  |
| GS6262-45 |  | GS6262-88 |  |
| GS6262-55 |  | GS6262-07 |  |
| GS6262-58 |  | GS6262-76 |  |
| GS6262-60 |  | GS6262-74 |  |
| GS6262-62 |  | GS6262-16 |  |

Example 3 - Staining of Reticulocytes with ReticRed1 in Isotonic Saline Solution ISOFLOW™

A blood sample procured from a local hospital was used to determine the staining kinetics of ReticRed1 (prepared as described in Examples 1A and D) alone. The blood sample was first analyzed by two independent reference automated methods: the reticONE™ method [described in Example 1G] and the New Methylene Blue method, a commercial method that is available for use in Gen*S™ instruments (Beckman Coulter). The reticONE™ reference method gave a retic percentage of 7% and the Gen*S™ method gave a retic percentage of 6.7%.

For measurements using ReticRed1, 1 µl of whole blood was added to 1 ml of an isotonic saline solution containing a 5 µM ReticRed1 (diluted from the 5 mM stock solution described in Example 1C) solution and incubated for 10 and 40 minutes. The sample was then analyzed in a XL™ flow cytometer modified to incorporate a red HeNe laser (632.8 nm) as described in Example 1E. When whole blood was treated with ReticRed1 and analyzed in flow, red fluorescence in the 660 nm band resulted from a population of red cells, and indicated the presence of nucleic acid bound to ReticRed1 in such cells. This fluorescence is due to reticulocytes stained with ReticRed1. The flow cytometry data can be summarized according to incubation durations as follows: (a) 10 minutes: Total count 42660, Red Cells 32841, Reticulocytes 801, calculated retic percentage 2.4% (b) 40 minutes: Total count: 46199, Red cells 39713, Reticulocytes 2310, calculated retic percentage 5.8%. See, Figs. 2A and 2B.

These results clearly demonstrate that the ReticRed1 dye alone requires a relatively long period of time to effectively stain the intracellular RNA.

Example 4 - Rapid Staining of Reticulocytes with ReticRed1 using Whole Blood Samples

Although Example 3 above demonstrated that the dye ReticRed1 in an isotonic saline solution takes a relatively long time to stain reticulocytes, we found that using this dye to stain reticulocytes in presence of additional reagent compositions of this invention can reduce the incubation time for the staining

significantly. Runs using four separate blood samples are presented to demonstrate rapid staining of reticulocytes by the dye ReticRed1.

*A. Run 1*

1 µl of whole blood (from the same donor whose blood was used to conduct the analyses described in example 3 above) was added to 1ml of a solution comprising a sphering reagent that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye ReticRed1 at a concentration of 5µM, and a solution of p-toluenesulfonic acid monohydrate at a final concentration of 5 µM, and incubated for about 1 minute. The sample was then analyzed in a XL™ flow cytometer modified to incorporate a red HeNe laser (632.8 nm). The sphering reagent is a solution comprising about 20 µg/ml Dodecyl-β-D-maltoside and 0.05% Proclin 300 in PBS at about pH 7.4 and about 290 mOsm.

The sample so stained with ReticRed1 was then analyzed in a flow cytometer using 632.8 nm excitation from a HeNe laser. Bright red fluorescence in the 660 nm band resulted from the reticulocytes. The results of this experiment are shown in Fig. 3. The distribution of the cells in this dotplot, showing forward scatter versus fluorescence, is consistent with the distribution of mature red cells and reticulocytes previously shown by Tanke et. al. [cited above], in relation to their work on fluorescence based reticulocyte measurements. The percentage of reticulocytes enumerated by the present method was 7.3%.

*B. Run 2*

1 µl of whole blood from another abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 4A, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye ReticRed1 at a concentration of 5µM, and a solution of p-toluenesulfonic acid monohydrate at a final concentration of 5 µM, and incubated for about 1 minute. The sample was then analyzed in a XL™ flow cytometer modified to incorporate a red HeNe laser (632.8 nm).

The sample so stained with the dye ReticRed1 was then analyzed in a flow cytometer using 632.8 nm excitation from a HeNe laser. The result of this

experiment is shown in Fig. 4. The distribution of the cells in this forward scatter versus fluorescence dotplot clearly shows the reticulocytes with high fluorescence intensity. The percentage of reticulocytes enumerated by this experiment was 7.5 %. An independent reference value for reticulocyte percentage in this sample was 8%.

*C. Run 3*

1 µl of whole blood from a normal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 4A, Run 1 above) that spheres red cells, detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye ReticRed1 at a concentration of 5µM, and a solution of p-toluenesulfonic acid monohydrate at a final concentration of 5 µM, and incubated for about one minute. The sample was then analyzed in a XL™ flow cytometer modified to incorporate a red HeNe laser (632.8 nm). The blood sample so stained with the dye ReticRed1 was analyzed in flow using 632.8 nm excitation from a HeNe laser. The result of this experiment is shown in Fig. 5. The reticulocyte percentage enumerated by this experiment was 0.82%. An independent reference measurement of the blood from the same donor using new methylene blue in a commercially available clinical hematology analyzer gave retic percentage of 0.95%.

*D. Run 4*

1 µl of abnormal whole blood from a donor was added to 1ml of a solution comprising a sphering reagent (described in Example 4A, Run 1 above) that spheres red cells, detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye ReticRed1 at a concentration of 5µM, and a solution of sodium p-toluenesulfonate at a final concentration of 5 µM, and incubated for about one minute. The sample was then analyzed in a XL™ flow cytometer modified to incorporate a red HeNe laser (632.8 nm).

The sample blood so stained with ReticRed1 was then analyzed in flow cytometer using 632.8 nm excitation from a HeNe laser. The result of this experiment is shown in Fig. 6. The reticulocyte percentage enumerated by this experiment was 13.7%. An independent reference method for this same sample gave a retic percentage value of 13%.

Example 5 - Rapid Staining of Reticulocytes with ReticRed3 using Whole Blood Samples

*A. Run 1*

2 µl of whole blood from an abnormal donor having a high reticulocyte count was added to 1ml of a solution comprising a sphering reagent (described in Example 4A, Run 1 above) that spheres the red cells, the detergent Igepal® (Sigma CA-630) at a concentration of 0.01%, the dye ReticRed1 at a concentration of 5µM, and a solution of sodium p-toluenesulfonate at a final concentration of 50 µM, and was gently mixed/incubated for about a second. The mixture was then aspirated into the XL™ flow cytometer containing the red HeNe laser for analysis.

The result of this experiment is shown in Fig. 7, where the reticulocytes are distinguished from the mature red blood cells by the higher fluorescence intensities associated with them. The percentage of reticulocytes enumerated by this experiment was 13.8 %. An independent reference value for reticulocyte percentage measured for this sample using the conventional Retic-Count™ reticulocyte enumeration method (using Thiazole orange excited at 488 nm) in a FACSCAN flow cytometer was 13.3%.

*B. Run 2*

2 µl of whole blood from an abnormal donor having low reticulocyte counts was added to 1ml of a solution comprising a sphering reagent (described in Example 4A, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye ReticRed1 at a concentration of 5µM, and a solution of sodium p-toluenesulfonate at a final concentration of 50 µM, and was gently mixed/incubated for about a second. The

mixture was then aspirated into the XL™ flow cytometer containing the red HeNe laser for analysis.

The result of this experiment is shown in Fig. 8. The percentage of reticulocytes enumerated by this experiment was 1 %. An independent reference value for reticulocyte percentage measured for this sample using the conventional Retic-Count™ reticulocyte enumeration method (Thiazole orange excited at 488 nm) in a FACSCAN flow cytometer was 1.3%.

Example 6 - Comparison of fluorescence intensities the dyes of present invention in bound and unbound states

To compare the relative fluorescence intensities of the dyes in bound and unbound states, fluorescence measurements were conducted in a spectrofluorometer by exciting each dye solution at 633 nm under appropriate conditions, recording the fluorescence spectrum of each sample, and measuring the intensities at the peak of these fluorescence spectra.

To measure the fluorescence intensities of the dye solution in absence of DNA or RNA (i.e., unbound state), first a stock solution of each compound in DMSO was prepared (as described in Example 1D). A portion of the stock solution for the Dye-1 was diluted in PBS to obtain a dye concentration of 2 µl. Next, 1 ml of this dilute solution was placed in a glass cuvette and fluorescence spectrum of this solution was measured by a spectrofluorometer using an excitation wavelength of 633 nm.

Next, 1ml of a solution of free Torula yeast RNA (Sigma) dissolved in PBS at a concentration of 1 mg/ml, and a measured volume of the dye stock solution of the Dye-1 was mixed to obtain a dye concentration of 2 µM in the RNA solution. The fluorescence spectrum of this dye solution in presence of RNA was measured by illuminating the sample at 633 nm. Fig. 9A shows the comparison of the fluorescence spectra of the dye Dye-1 in PBS solution with (see the upper curve) and without RNA (see the lower curve) present in the solution.

For fluorescence measurements in DNA, 1ml of a solution of free Calf thymus DNA (Sigma) dissolved in PBS at a concentration of 0.5 mg/ml, and a

measured volume of the dye stock solution was mixed to obtain a dye concentration of 2 μM in the RNA solution. Next, the fluorescence spectrum of the dye solution was measured by illuminating the sample at 633 nm. Fig. 9B shows the comparison of the fluorescence spectra of the dye Dye-1 in PBS solution with (see the upper curve) and without DNA (see the lower curve) present in the solution.

In the same manner, the experiments described above were repeated for the dye Dye-2 through Dye-6 (see Figs. 9C- and 9L).

Table 3 compares the relative fluorescence intensities of the dyes of the present invention, Dye-1 and Dye-2 (concentration 2 μM), when they are not bound to RNA (unbound to RNA), when they are in presence of RNA (bound to RNA), and when they are in presence of DNA (bound to DNA). These intensities were obtained at the peak of the respective fluorescence spectra as described above.

**Table-3**

| DYE (conc. 2 μm) | Fluorescence Intensity | | |
|---|---|---|---|
| | Unbound to RNA | Bound to RNA 1mg/ml | Bound to DNA 0.5mg/ml |
| Dye-1 | 10 | 825 | 578 |
| Dye-2 | 18 | 560 | 760 |

Table 4 compares the fluorescence intensities of the Group-III dyes (Dye-3 through Dye-6) of the present invention, when they are not bound to RNA (unbound to RNA) and when they are in presence of RNA (bound to RNA). The intensities were measured at the peak of the emission spectra.

Table-5 compares the fluorescence intensities of the Group-III dyes (Dye-3 through Dye-6) of the present invention, when they are not bound to DNA (unbound to DNA) and when they are in presence of DNA (bound to DNA). The intensities were measured at the peak of the emission spectra.

**Table-4**

| DYE (conc. 2 μm) | Fluorescence Intensity | |
|---|---|---|
| | Unbound to RNA | Bound to RNA 1mg/ml |
| Dye-3 | 10 | 328 |
| Dye-4 | 12 | 370 |
| Dye-5 | 10 | 420 |
| Dye-6 | 6 | 410 |

**Table-5**

| DYE | Fluorescence Intensity | |
|---|---|---|
| | Unbound to DNA | Bound to DNA 0.5 mg/ml |
| Dye-3 | 15 | 400 |
| Dye-4 | 12 | 440 |
| Dye-5 | 10 | 660 |
| Dye-6 | 4 | 380 |

Example 7 - Rapid staining of reticulocytes with Dye-1 using whole blood samples

We found that the dye Dye-1 can pass through the cell membrane of reticulocytes rapidly in presence of the reagent compositions of this invention, thus reducing the incubation time for the staining significantly. Experiments using three separate blood samples are presented to demonstrate rapid staining of reticulocytes by the dye Dye-1. We refer to these three experiments as Run 1, Run 2 and Run 3 respectively.

*A. Run 1*

1 μl of whole blood from a normal donor was added to 1ml of a solution comprising a sphering reagent that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye Dye-1 at a concentration of 5μM, and aspirated, immediately thereafter, into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis. The sphering reagent is a solution comprising about 20 μg/ml Dodecyl-β-D-maltoside and 0.05% Proclin 300 in PBS at about pH 7.4 and about 290 mOsm.

Fig. 10A. shows the fluorescence from reticulocytes relative to the mature red blood cells for this sample. The percentage of reticulocytes enumerated by the present method was 1.9%, which was within the expected normal range.

*B. Run 2*

1 µl of whole blood from an abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 7, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, and the dye Dye-1 at a concentration of 5µM. Immediately thereafter, the mixture was aspirated into an XL™ flow cytometer modified to incorporate a red HeNe laser (632.8 nm), for analysis.

Fig. 10B shows the fluorescence from reticulocytes relative to the mature red blood cells for this sample. The distribution of the cells in this forward scatter versus fluorescence dotplot clearly shows the reticulocytes with relatively high fluorescence intensity. The percentage of reticulocytes enumerated by this experiment was 10.6% An independent reference value for reticulocyte percentage in this sample was 12%.

*C. Run 3*

1 µl of whole blood from another abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 7, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, and the dye Dye-1 at a concentration of 5µM. Immediately thereafter, the mixture was aspirated into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis.

Fig. 10C shows the fluorescence from reticulocytes relative to the mature red blood cells for this sample. The distribution of the cells in this forward scatter versus fluorescence dotplot clearly shows the reticulocytes with high fluorescence intensity. The percentage of reticulocytes enumerated by this experiment was 26.3%. An independent reference value for reticulocyte percentage in this sample was 27%.

Example 8 - Rapid staining of reticulocytes with Dye-2 using whole blood samples

We found that Dye-2 can also rapidly pass through the cell membrane of reticulocytes in presence of the reagent compositions of this invention, thus reducing the incubation time for the staining significantly. Experiments using three different blood samples are presented to demonstrate rapid staining of reticulocytes by the dye Dye-2. We refer to these three experiments as Run 4, Run 5 and Run 6 respectively.

*A. Run 4*

1 µl of whole blood from a normal donor was added to 1ml of a solution comprising a sphering reagent that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye Dye-2 at a concentration of 5µM, and aspirated, immediately thereafter, into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis. The sphering reagent is a solution comprising about 20 µg/ml Dodecyl-β-D-maltoside and 0.05% Proclin 300 in PBS at about pH 7.4 and about 290 mOsm.

Fig. 11A shows the fluorescence from reticulocytes relative to the mature red blood cells for this sample. The percentage of reticulocytes enumerated by the present method was 1.8%.

*B. Run 5*

1 µl of whole blood from an abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 7, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, and the dye Dye-2 at a concentration of 5µM. Immediately thereafter, the mixture was aspirated into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis.

Fig. 11B shows the fluorescence from reticulocytes relative to the mature red blood cells for this sample. The distribution of the cells in this forward scatter versus fluorescence dotplot clearly shows the reticulocytes with high fluorescence intensity. The percentage of reticulocytes enumerated by this

experiment was 12.5%. An independent reference value for reticulocyte percentage in this sample was 12%.

*C. Run 6*

1 µl of whole blood from a high retic abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 7, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, and the dye Dye-2 at a concentration of 5µM. Immediately thereafter, the mixture was aspirated into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis.

Fig. 11C shows the fluorescence from reticulocytes relative to the mature red blood cells for this sample. The distribution of the cells in this forward scatter versus fluorescence dotplot clearly shows the reticulocytes with high fluorescence intensity. The percentage of reticulocytes enumerated by this experiment was 24 %. An independent reference value for reticulocyte percentage in this sample was 27%.

Example 9 - Rapid staining of reticulocytes with Dye-3 using whole blood samples

*A. Run 7*

1 µl of whole blood from a normal donor was added to 1ml of a solution comprising a sphering reagent that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, the dye Dye-3 at a concentration of 5µM, and aspirated, immediately thereafter, into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis. The sphering reagent is a solution comprising about 20 µg/ml Dodecyl-β-D-maltoside and 0.05% Proclin® 300 in PBS at about pH 7.4 and about 290 mOsm. The percentage of reticulocytes enumerated by the present method was 2.3% (see Fig. 4A).

*B. Run 8*

1 µl of whole blood from an abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 7, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, and the dye Dye-3 at a concentration of 5µM. Immediately thereafter, the mixture was aspirated into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis. The percentage of reticulocytes enumerated by this experiment was approximately 14.6% (see Fig. 4A). An independent reference value for reticulocyte percentage in this sample was 12% .

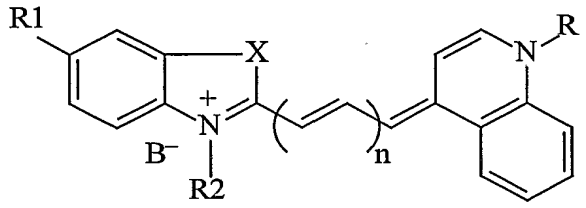
*C. Run 9*

1 µl of whole blood from another abnormal donor was added to 1ml of a solution comprising a sphering reagent (described in Example 7, Run 1 above) that spheres the red cells, the detergent Igepal (Sigma CA-630) at a concentration of 0.01%, and the dye Dye-3 at a concentration of 5µM. Immediately thereafter, the mixture was aspirated into an XL™ flow cytometer, modified to incorporate a red HeNe laser (632.8 nm), for analysis. The percentage of reticulocytes enumerated by this experiment was approximately 24% (see Fig. 12A). An independent reference value for reticulocyte percentage in this sample was 27%.

All publications cited in this specification are incorporated herein by reference herein. While the invention has been described with reference to a particularly preferred embodiment, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A dye having the formula:




wherein,
n is 0, 1, 2, or 3; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOH$, wherein m is 0, 1, 2, or 3; X is O, S, or $C(CH_3)_2$; R is $CH_3$, $CH(CH_3)_2$, $CH_2CH_2OH$, alkyl, alkylsulfonate, or hydroxyalkyl and B- is a counteranion.

2. The dye according to claim 1, wherein n is 1, $R_1$ is H, R is $CH_2CH_2OH$, R2 is $CH_2CH_2OH$, and X is S.

3. The dye according to claim 1, wherein n is 1, $R_1$ is H, R is $CH(CH_3)_2$, $R_2$ is $CH_2CH_2OH$ and X is S.

4. The dye according to claim 1, wherein n is 1, $R_1$ is H, R is $CH_3$, $R_2$ is $CH_2CH_2OH$, and X is S.

5. The dye according to claim 1, wherein n is 1, $R_1$ is $CH_3$, R is $CH_3$, $R_2$ is $CH_2CH_2OH$, and X is S.

6. The dye according to claim 1, wherein n is 1, $R_1$ is $CH_3$, R is $CH(CH_3)_2$, $R_2$ is $CH_2CH_2OH$, and X is S.

7. The dye according to claim 1, wherein n is 1, $R_1$ is $CH_3$, R is $CH_2CH_2OH$, $R_2$ is $CH_2CH_2OH$, and X is S.

8. A dye composition for staining nucleic acids comprising the dye of claim 1.

9. A dye composition for staining reticulocytes comprising the dye of claim 1.

10. The dye composition for staining reticulocytes according to claim 9 further comprising a surfactant.

11. The dye composition according to claim 9, further comprising a detergent in an amount of about 0 up to about 1%.

12. The dye composition according to claim 11, wherein said detergent is selected from the group consisting of octyphenoxypoly (ethyleneoxy) ethanol, ethoxylated octylphenol, linear alcohol alkoxylates.

13. The dye composition according to claim 9 further comprising a sulfonic acid or a salt thereof.

14. The dye composition of claim 10, wherein said surfactant comprises a non-ionic surfactant.

15. The dye composition of claim 14, wherein said non-ionic surfactant is selected from the group consisting of dodecyl-β-D-maltoside, N, N-bis[3-D-glucon-amidopropyl] cholamide, polyoxypropylene-polyoxyethylene block copolymer, N-Tetradecyl-β-D-maltoside, Daconyl-N-methyl-glucamide, n-

Dodecyl-β-D-glucopyranoside, n-Decyl-β-D-glucopyranoside, polyethylene glycol ester of stearic acid, ethoxylated cocomonoglyceride, octyphenoxypoly (ethyleneoxy) ethanol, ethoxylated octylphenol, and linear alcohol.

16. The dye composition of claim 10, wherein said surfactant comprises a cationic surfactant.

17. The dye composition of claim 16, wherein said cationic surfactant is selected from the group consisting of coco hydroxyethyl imidazoline, lauryltrimethylammonium chloride, decyltrimethylammonium bromide, and octyltrimethylammonium bromide.

18. The dye composition of claim 10, wherein said surfactant comprises an anionic surfactant.

19. The dye composition of claim 18, wherein said anionic surfactant is selected from the group consisting of ammonium perfluoralkyl carboxylate, sodium lauroyl myristoyl lactylate.

20. The dye composition of claim 10, wherein said surfactant comprises a zwitterionic surfactant.

21. The dye composition of claim 20, wherein said zwitterionic surfactant is selected from the group consisting of lauramidopropyl betaine, N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, cocoamidopropylbetaine, cocoamidosulfobetaine, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate.

22. The dye composition of claim 9, further comprising a preservative.

23. The dye composition according to claim 22, wherein said preservative comprises 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one.

24. The dye composition of claim 13, wherein said sulfonic acid is p-toluenesulfonic acid .

25. The dye composition according to claim 24, wherein concentration of said p-toluenesulfonic acid is about 0.01 to about 250 μM.

26. The dye composition according to claim 13, wherein said sulfonate salt is selected from the group consisting of sodium p-toluenesulfonate, silver p-toluenesulfonate, zinc p-toluenesulfonate.

27. The dye composition according to claim 26, wherein concentration of said sodium p-toluenesulfonate is about 0.01 to about 250 μM.

28. A method for staining nucleic acid comprising the step of contacting a sample containing nucleic acid with the dye according to claim 1.

29. The method according to claim 28, wherein said sample comprises cells, and wherein said sample is contacted by the dye in the presence of a surfactant.

30. The method according to claim 28, wherein said sample comprises whole blood.

31. A method for analysis of reticulocytes comprising the steps of: (a) contacting a sample containing reticulocytes with a compound according to claim 1 such that the reticulocytes are stained by the said compound; and (b) analyzing the stained reticulocytes by flow cytometry to detect the presence of reticulocytes.

32. The method according to claim 31, wherein said analysis by flow cytometry includes measurement of one fluorescence parameter and at least one parameter selected from the group consisting of light scatter, optical absorption, axial light loss, DC electrical impedance, and radio frequency (RF) conductivity and combinations thereof.

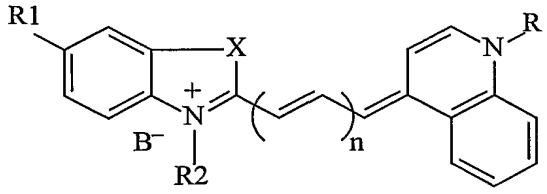
33. A method for facilitating transport of dye composition through a cell membrane comprising the steps of

(a) contacting a sample comprising a cell with a dye according to claim 1, a surfactant, and a sulfonic acid or salt thereof, and

(b) incubating the said mixture for up to about one minute.

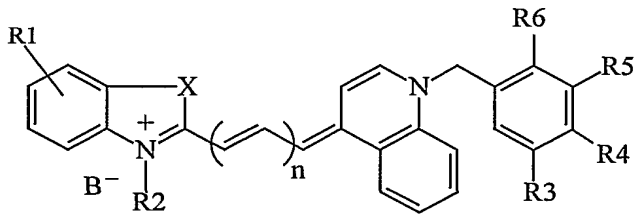
34. The method according to claim 33, wherein said incubation is conducted in the temperature range of about 20°C to about 40°C.

35. A dye having the formula:




wherein, n is 0, 1, 2, or 3; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOAc$, wherein m is 0, 1, 2, or 3; X is O, S, or $C(CH_3)_2$; R is $CH_3$, $CH(CH_3)_2$, $CH_2CH_2OAc$, alkyl, alkylsulfonate, or hydroxyalkyl and B- is a counteranion.

36. A dye according to the formula:




wherein n is 0, 1, or 2; R1 is H, alkyl, or an alkoxy group; R2 is $CH_2(CH_2)_mOH$, or $CH_3$ ; X is O, S, or $C(CH_3)_2$; $B^-$ is a counter anion, and R3, R4, R5, R6 are various substituents as represented by the formulae for the specific compounds Dye-3 through Dye-6.

37. The dye according to claim 35, wherein n = 1; $R_1$ = H; R = $CH_3$, R2 = $CH_2CH_2OAc$; X = S, and $B^-$ is $Br^-$.

38. The dye according to claim 35, wherein n = 1, R1 = H, R2 = R = $CH_2CH_2OAc$, X = S, and $B^-$ is Br-.

39. The dye according to claim 36, wherein n= 1; X = S; $R_1$= $CH_2CH_2OH$; $R_2$ = $R_4$ = R5=H; R3=$CO.CH_3$; and $B^-$ is $Br^-$.

40. The dye according to claim 36, wherein n= 1; X = S; $R_1$= $CH_2CH_2OH$; $R_2$ = $R_4$ = R5=H; R3=$CO.CH_3$; and $B^-$ is $Br^-$.

41. The dye according to claim 36, wherein n= 1; X = S; $R_1$= $CH_2CH_2OH$; $R_2$= $R_3$= $R_4$= $R_5$= H; and $B^-$ is $Br^-$.

42. The dye according to claim 36, wherein n= 1; X = S; $R_1$= $CH_2CH_2OH$; $R_2$= $R_3$= $R_4$= H; $R_5$= $B(OH)_2$; and $B^-$ is $I^-$.

43. A dye composition for staining nucleic acids comprising the compound of claim 35 or claim 36 dissolved in a solvent.

44. A dye composition for staining blood cells comprising the compound of claim 35 or claim 36.

45. A dye composition for staining blood cells comprising the compound of claim 35 or claim 36, wherein the blood cells are nucleated red blood cells.

46. A dye composition for staining blood cells comprising the compound of claim 35 or claim 36, wherein the blood cells are reticulocytes.

47. The dye composition for staining reticulocytes according to claim 44 further comprising a surfactant.

48. The dye composition according to claim 44, further comprising a detergent in an amount of about 0% up to about 1%.

49. The dye composition according to claim 48, wherein said detergent is selected from the group consisting of octylphenoxypoly (ethyleneoxy) ethanol, ethoxylated octylphenol, and linear alcohol alkoxylates.

50. The dye composition according to claim 44, further comprising a sulfonic acid or a salt thereof.

51. The dye composition of claim 47, wherein said surfactant comprises a non-ionic surfactant.

52. The dye composition of claim 51, wherein said non-ionic surfactant is selected from the group consisting of dodecyl-β-D-maltoside, N, N-bis[3-D-glucon-amidopropyl] cholamide, polyoxypropylene-polyoxyethylene block copolymer, N-Tetradecyl-β-D-maltoside, Daconyl-N-methyl-glucamide, n-Dodecyl-β-D-glucopyranoside, n-Decyl-β-D-glucopyranoside, polyethylene glycol ester of stearic acid, ethoxylated cocomonoglyceride, octylphenoxypoly (ethyleneoxy) ethanol, ethoxylated octylphenol, and linear alcohol alkoxylates.

53. The dye composition of claim 47, wherein said surfactant comprises a cationic surfactant.

54. The dye composition of claim 53, wherein said cationic surfactant is selected from the group consisting of coco hydroxyethyl imidazoline, lauryltrimethylammonium chloride, decyltrimethylammonium bromide, and octyltrimethylammonium bromide.

55. The dye composition of claim 47, wherein said surfactant comprises an anionic surfactant.

56. The dye composition of claim 55, wherein said anionic surfactant is selected from the group consisting of ammonium perfluoralkyl carboxylate, sodium lauroyl myristoyl lactylate.

57. The dye composition of claim 47, wherein said surfactant comprises a zwitterionic surfactant.

58. The dye composition of claim 57, wherein said zwitterionic surfactant is selected from the group consisting of lauramidopropyl betaine, N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, cocoamidopropylbetaine, cocoamidosulfobetaine, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate.

59. The dye composition of claim 44, further comprising a preservative.

60. The dye composition according to claim 59, wherein said preservative comprises 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one.

61. The dye composition of claim 50, wherein said sulfonic acid is p-toluenesulfonic acid .

62. The dye composition according to claim 61, wherein concentration of said p-toluenesulfonic acid is about 0.01 to about 250 μM.

63. The dye composition according to claim 50, wherein said sulfonate salt is selected from the group consisting of sodium p-toluenesulfonate, silver p-toluenesulfonate, zinc p-toluenesulfonate.

64. The dye composition according to claim 63, wherein concentration of said sodium p-toluenesulfonate is about 0.01 to about 250 μM.

65. A method for staining nucleic acid comprising the step of contacting a sample containing nucleic acid with the dye according to claim 35 or claim 36.

66. The method according to claim 65, wherein said sample comprises blood cells.

67. The method according to claim 66, wherein said sample is contacted by the dye in the presence of a surfactant.

68. The method according to claim 66, wherein said sample is contacted by the dye after lysing the mature red blood cells and reticulocytes but preserving the nucleated red blood cells.

69. A method for analysis of reticulocytes comprising the steps of: (a) contacting a sample containing reticulocytes with a compound according to claim 35 or claim 36 such that the reticulocytes are stained by the said compound; and (b) analyzing the stained reticulocytes by flow cytometry to detect the presence of reticulocytes.

70. The method according to claim 69, wherein said analysis by flow cytometry includes measurement of one fluorescence parameter and at least one parameter selected from the group consisting of light scatter, axial light loss, DC electrical impedance, and radio frequency (RF) conductivity and combinations thereof.

71. A method for facilitating transport of dye composition through a cell membrane comprising the steps of

(a) contacting a sample comprising a cell with a dye according to claim 35 or claim 36, a surfactant, and a sulfonic acid or salt thereof, and

(b) incubating the said mixture for up to about one minute.

72. The method according to claim 67, wherein said incubation is conducted in the temperature range of about 20°C to about 40°C.

FIG. 1A

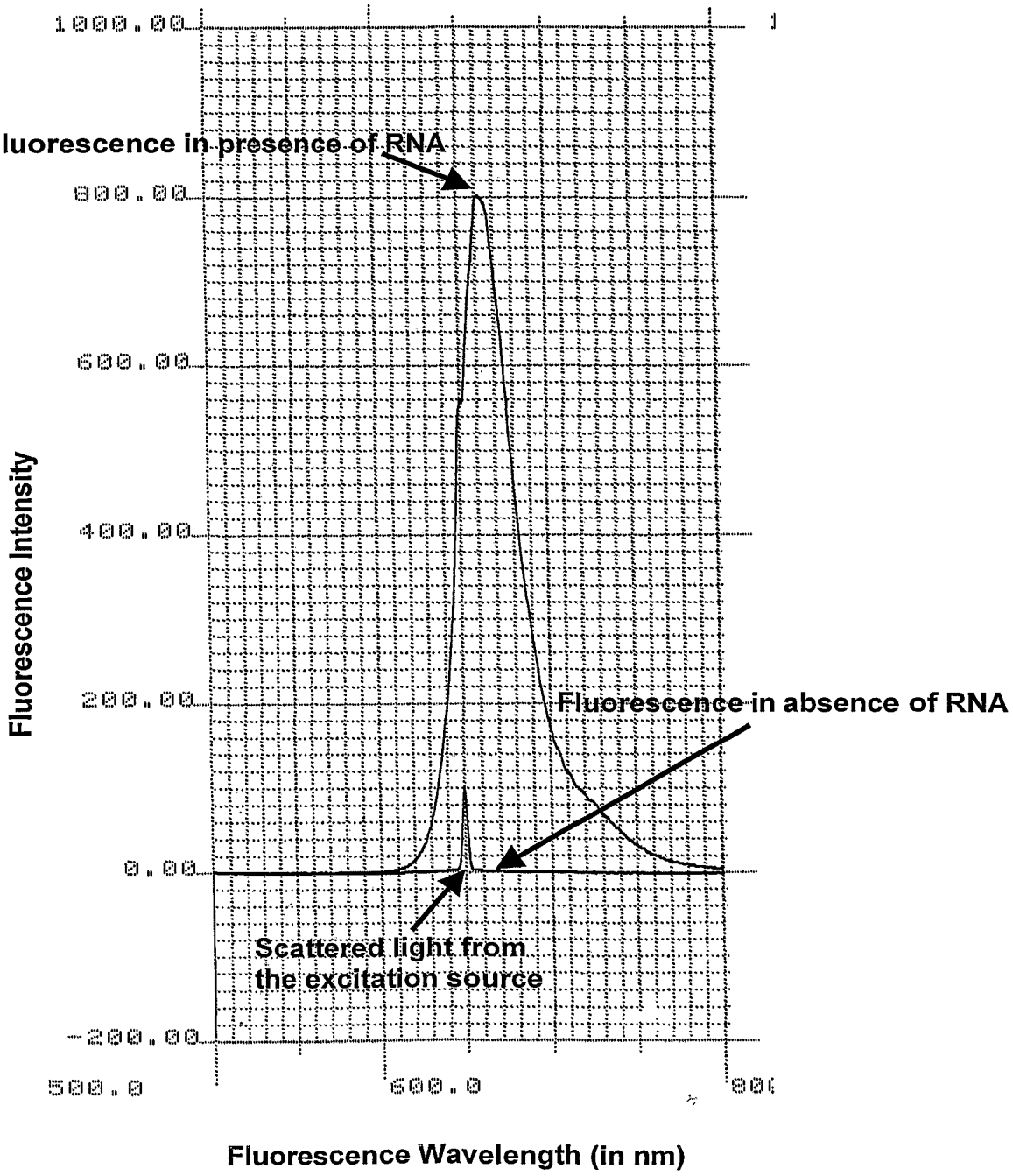

1000.00
800.00
600.00
400.00
200.00
0.00
-200.00
500.0
600.0
Fluorescence Intensity
Fluorescence Wavelength (in nm)
Fluorescence in presence of RNA
Fluorescence in absence of RNA
Scattered light from
the excitation source

FIG. 1B

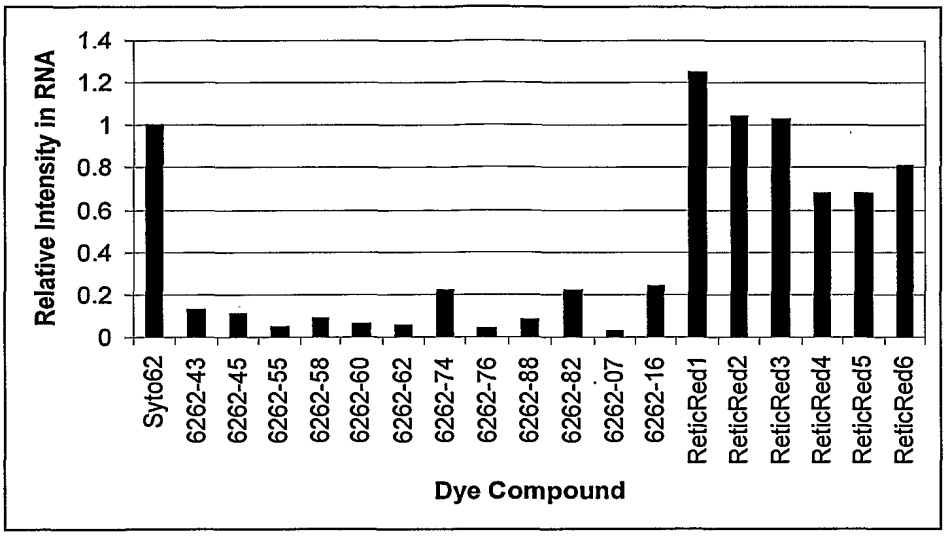
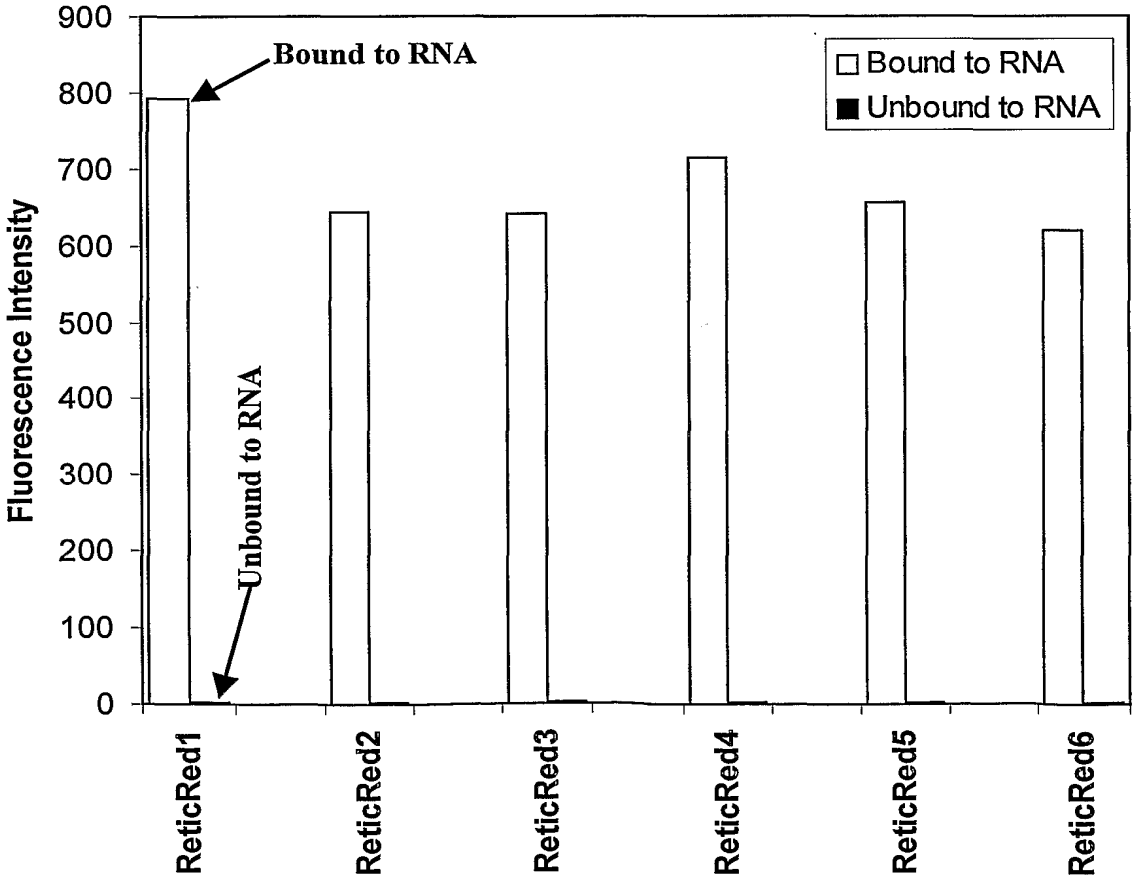

Relative Intensity in RNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Syto62
6262-43
6262-45
6262-55
6262-58
6262-60
6262-62
6262-74
6262-76
6262-88
6262-82
6262-07
6262-16
ReticRed1
ReticRed2
ReticRed3
ReticRed4
ReticRed5
ReticRed6
Dye Compound

FIG. 1C


Fluorescence Intensity
900
800
700
600
500
400
300
200
100
0
Bound to RNA
Unbound to RNA
Bound to RNA
Unbound to RNA
ReticRed1
ReticRed2
ReticRed3
ReticRed4
ReticRed5
ReticRed6

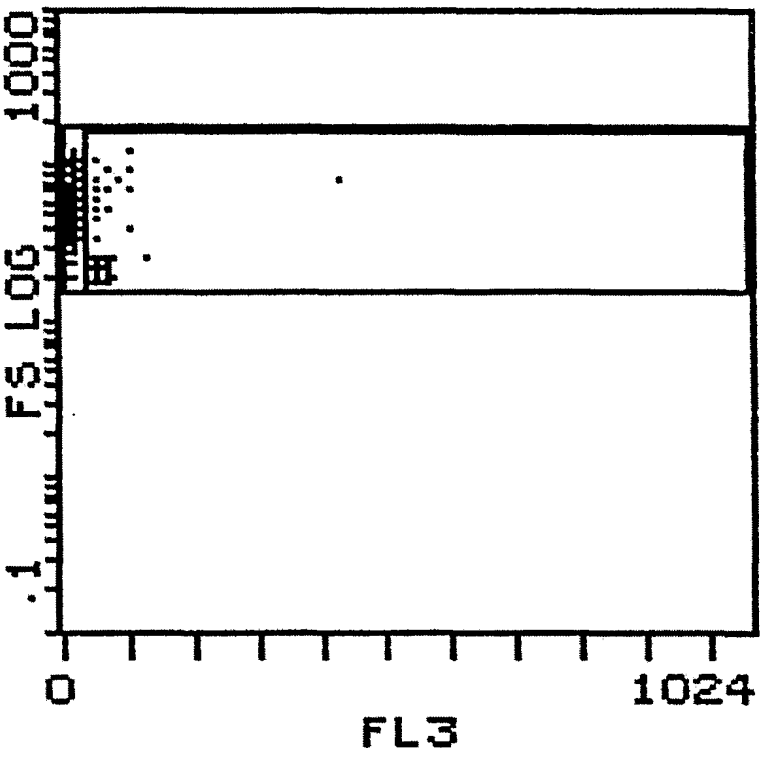



FIG. 2A

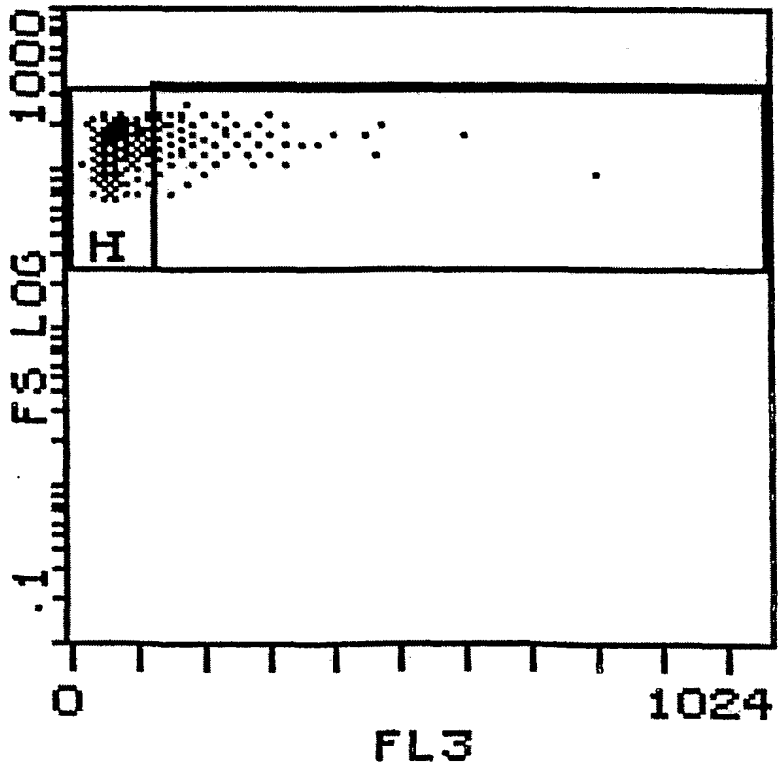



FIG. 2B

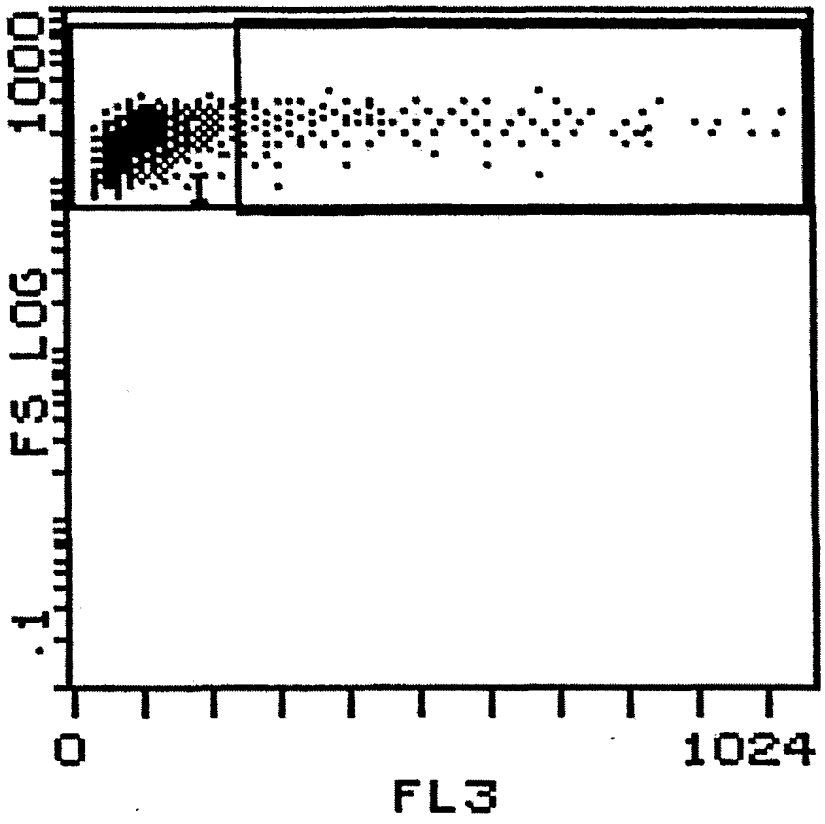



FIG. 3

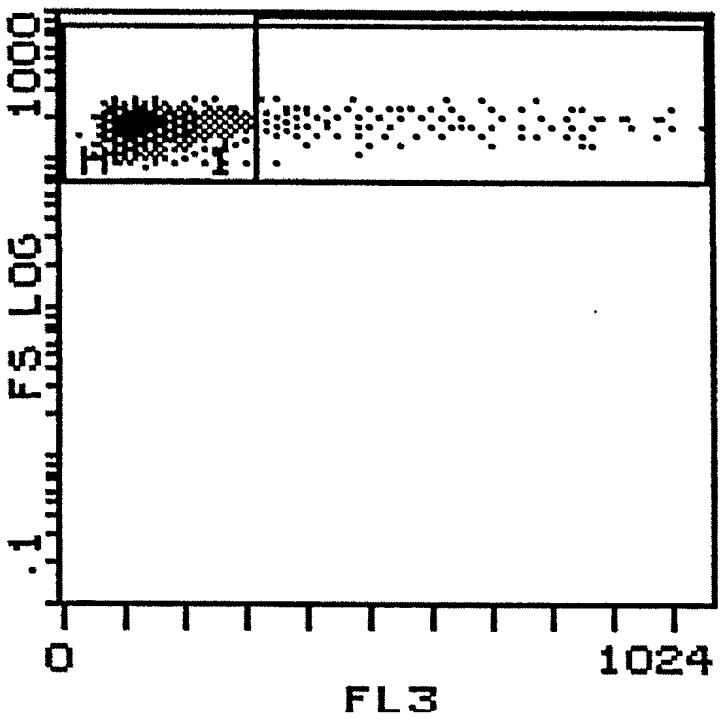



FIG. 4

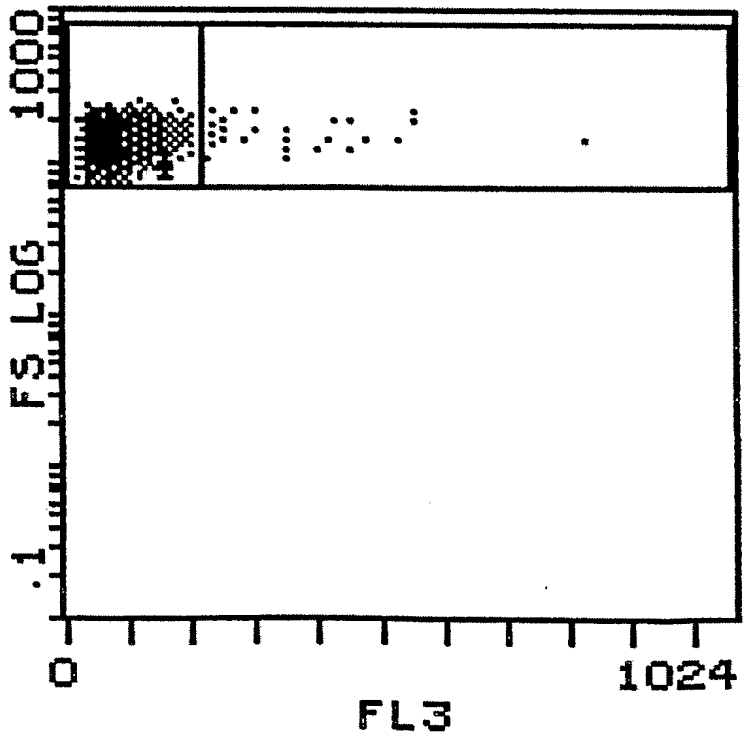



FIG. 5

FIG. 6

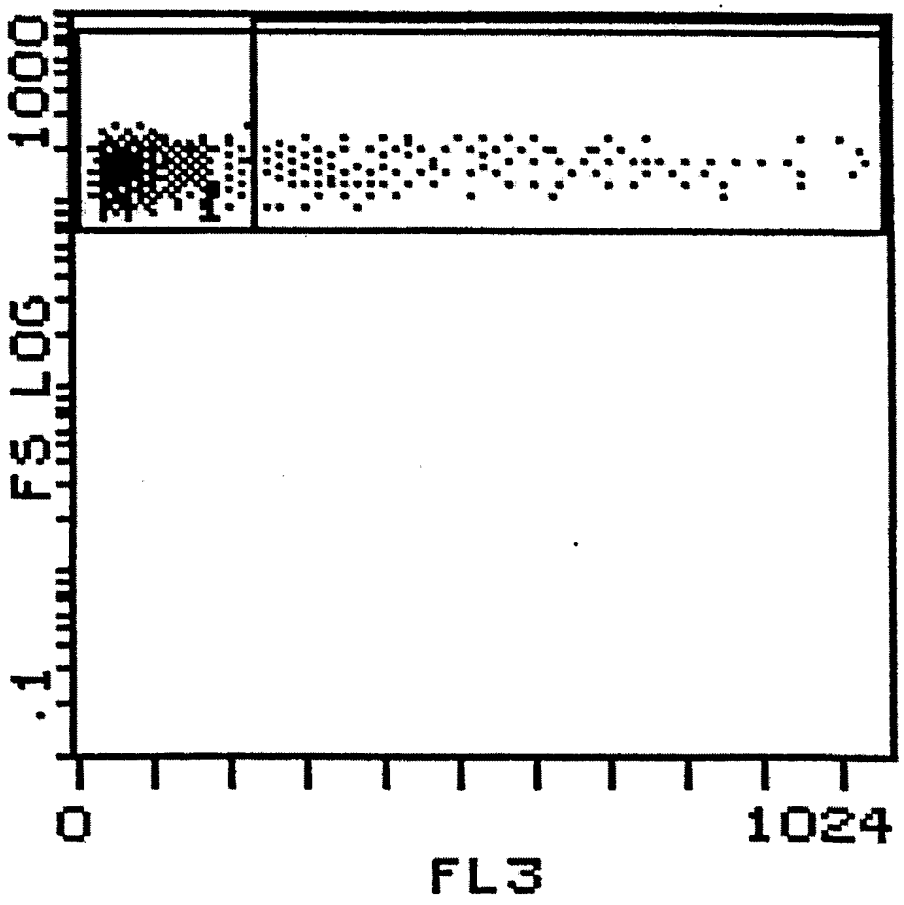


FIG. 7

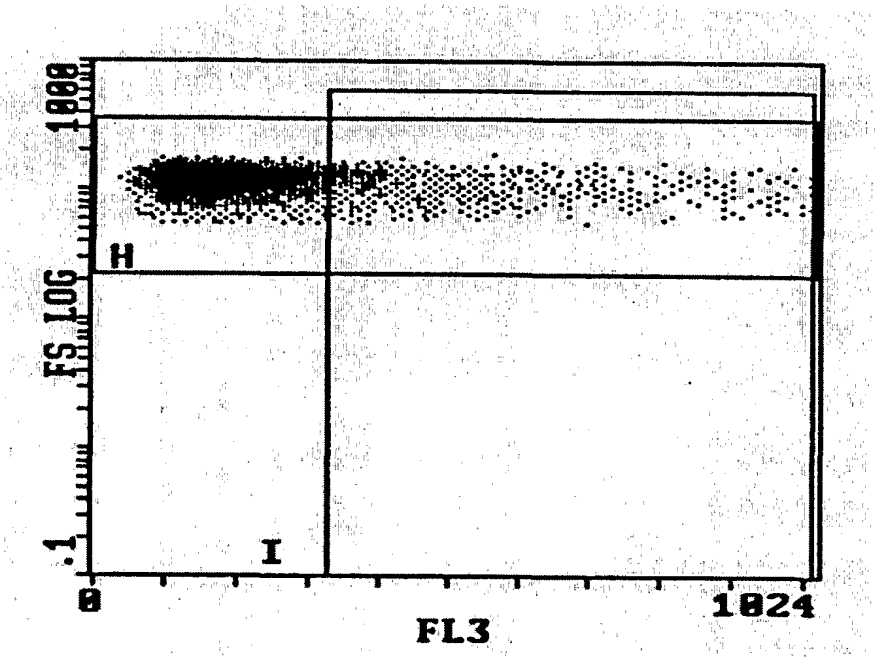
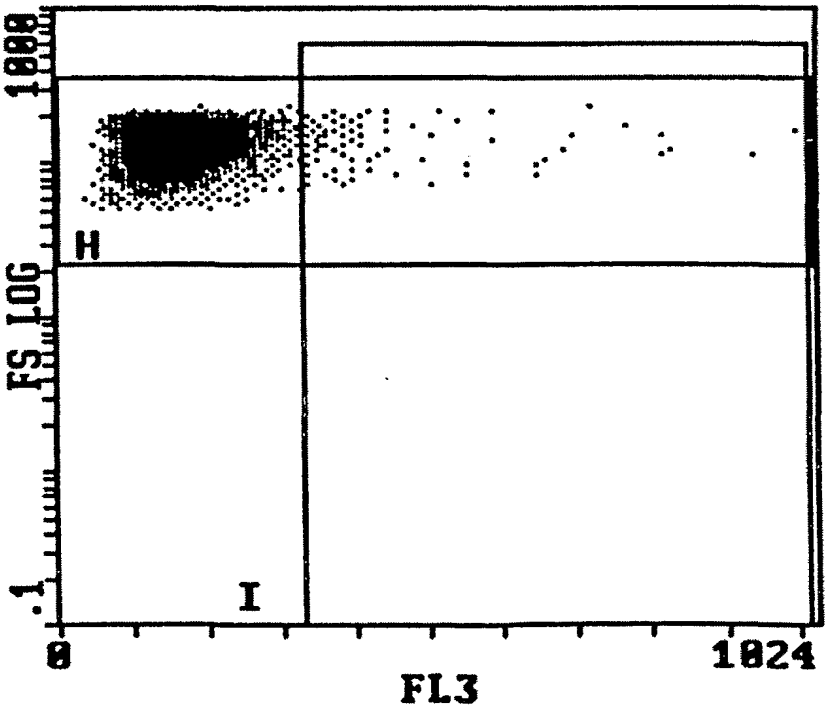



FIG. 8



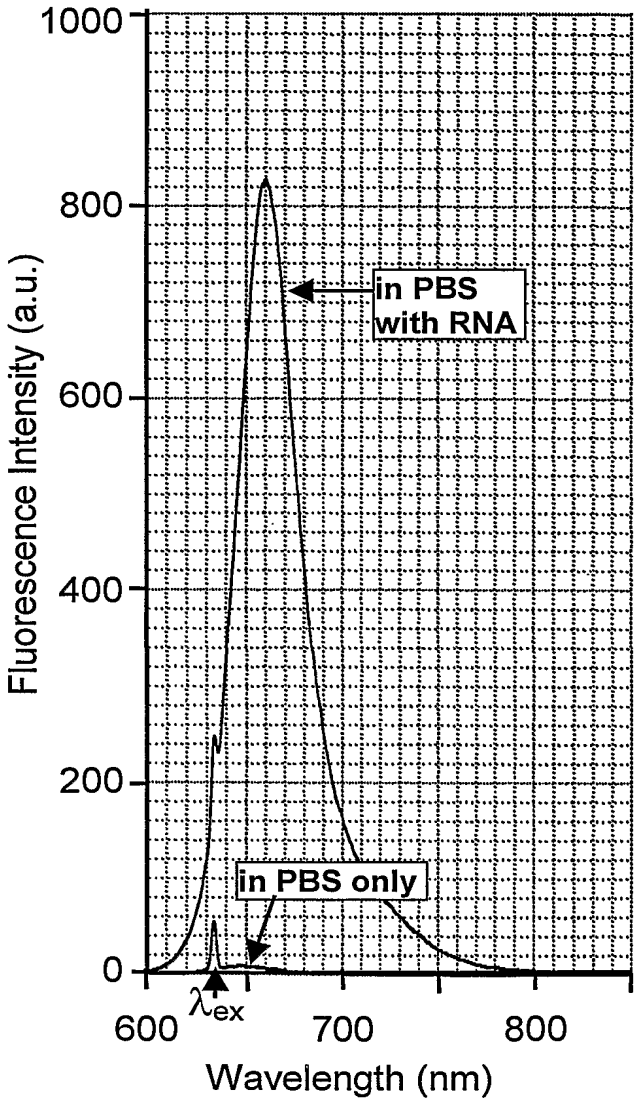

1000
800
600
400
200
0
Fluorescence Intensity (a.u.)
in PBS
with RNA
in PBS only
λex
600
700
800
Wavelength (nm)

FIG. 9A

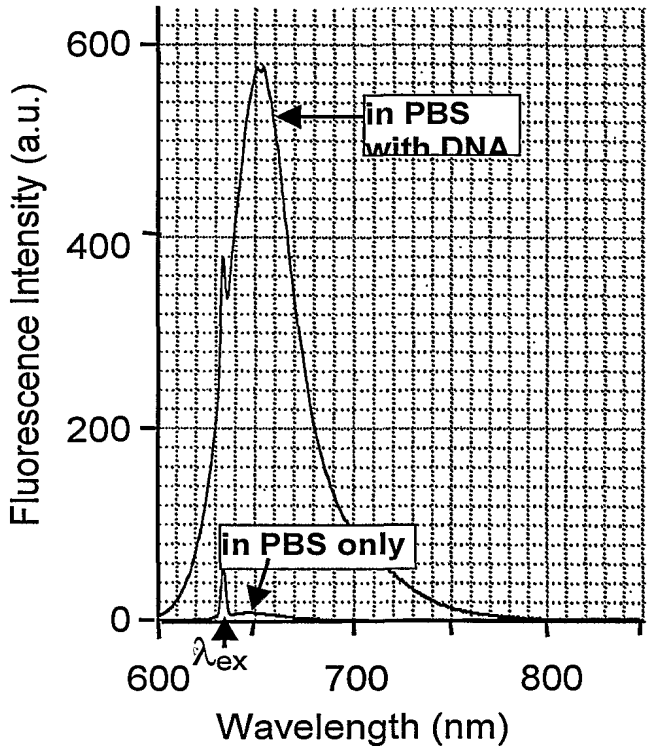

600
400
200
0
Fluorescence Intensity (a.u.)
in PBS with DNA
in PBS only
λex
600
700
800
Wavelength (nm)

FIG. 9B

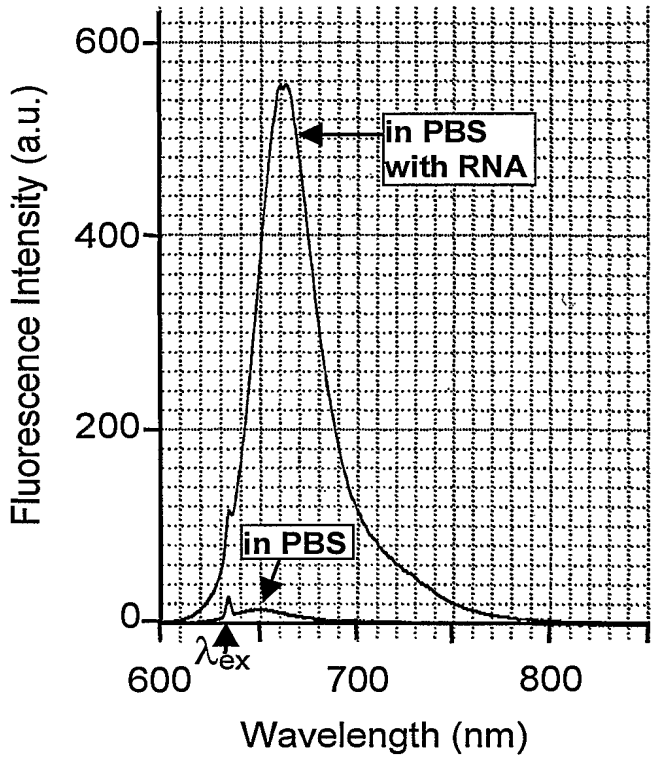

600
400
200
0
Fluorescence Intensity (a.u.)
in PBS
with RNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9C

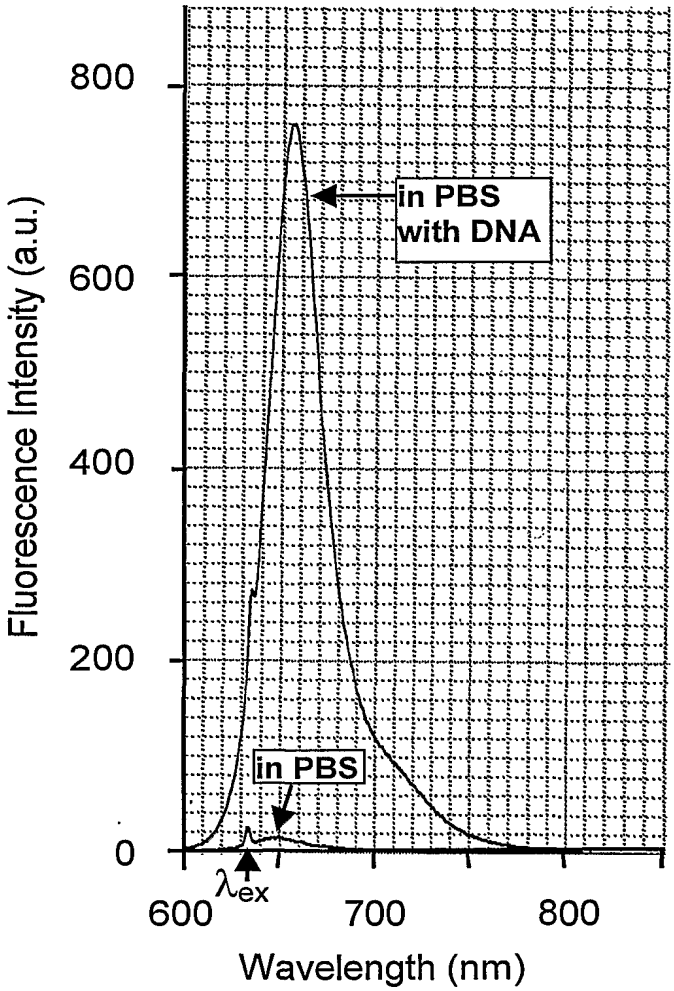

800
600
400
200
0
Fluorescence Intensity (a.u.)
in PBS
with DNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9D

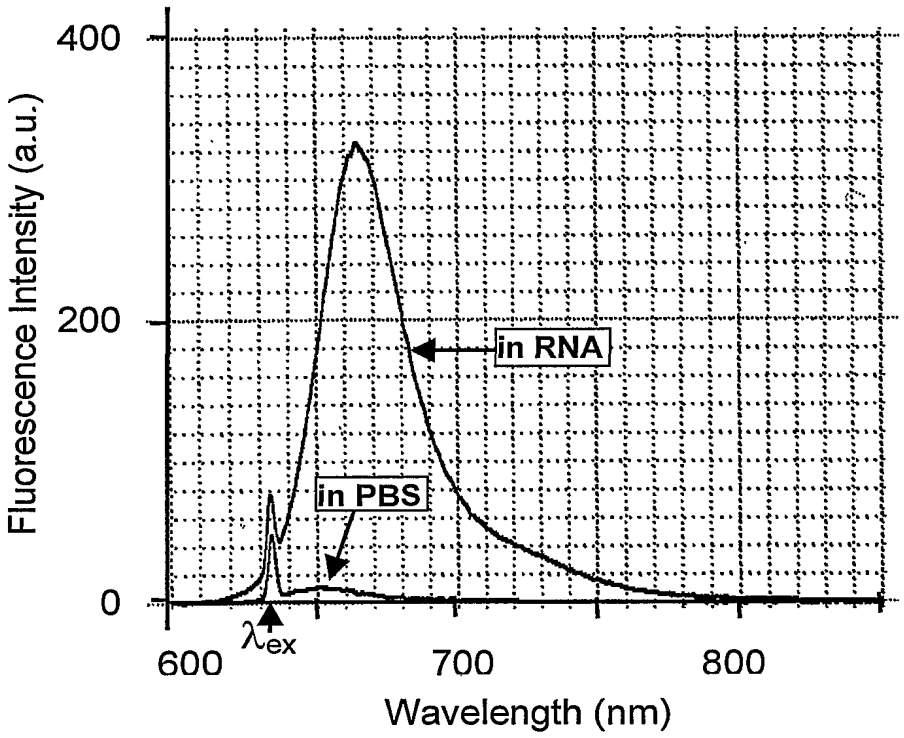

400
200
0
Fluorescence Intensity (a.u.)
in RNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9E

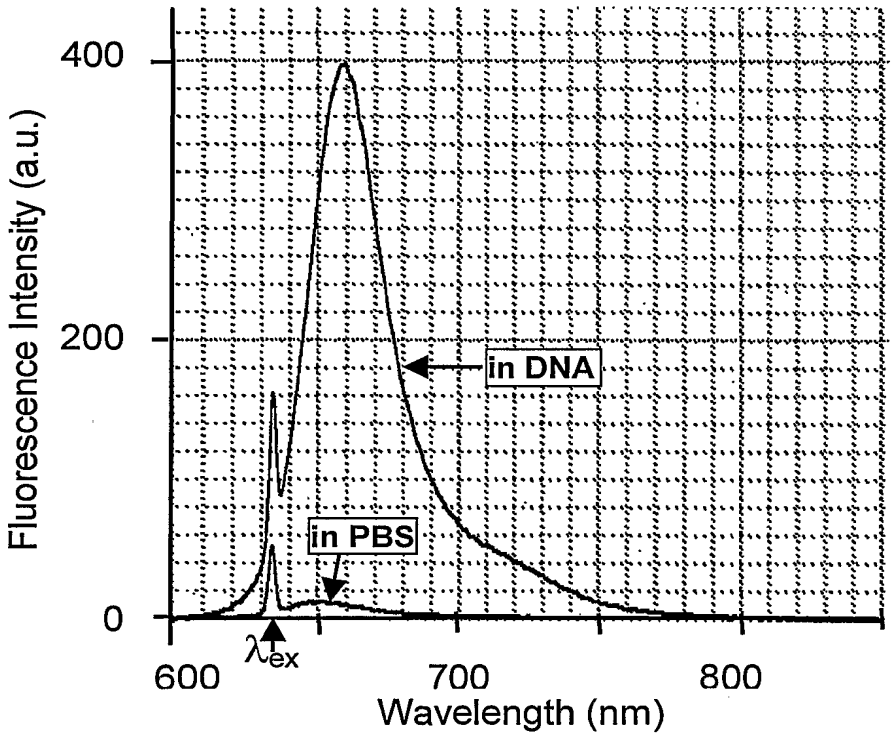

400
200
0
Fluorescence Intensity (a.u.)
in DNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9F

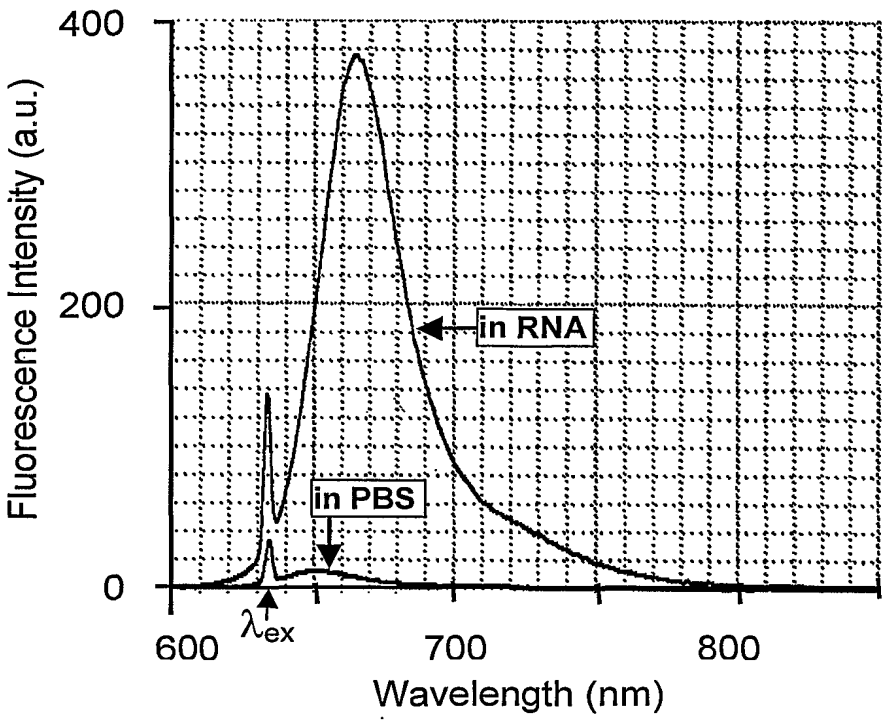

400
200
0
Fluorescence Intensity (a.u.)
in RNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9G

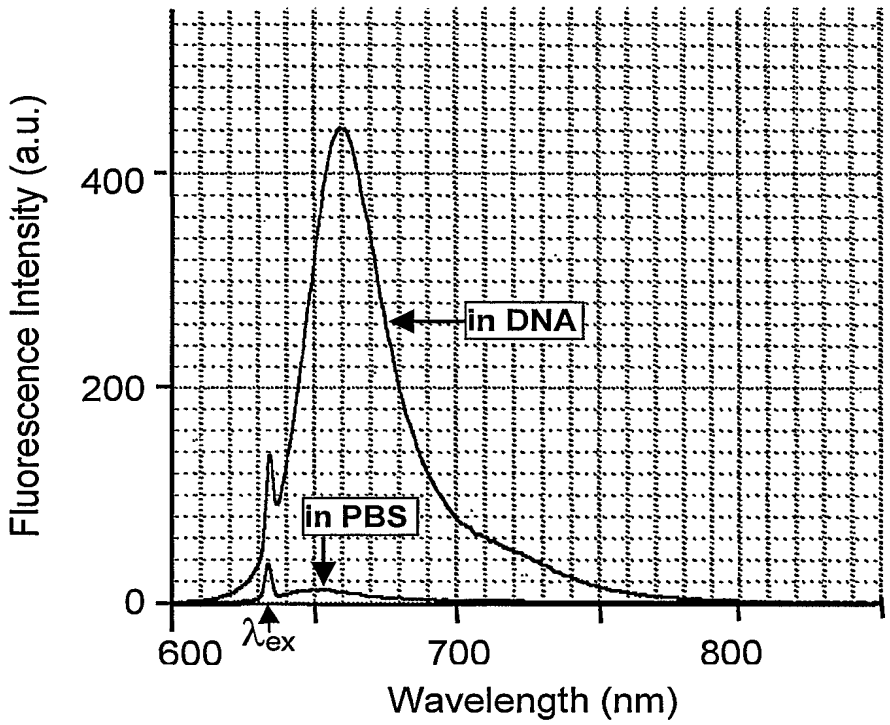

Fluorescence Intensity (a.u.)
400
200
0
in DNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9H

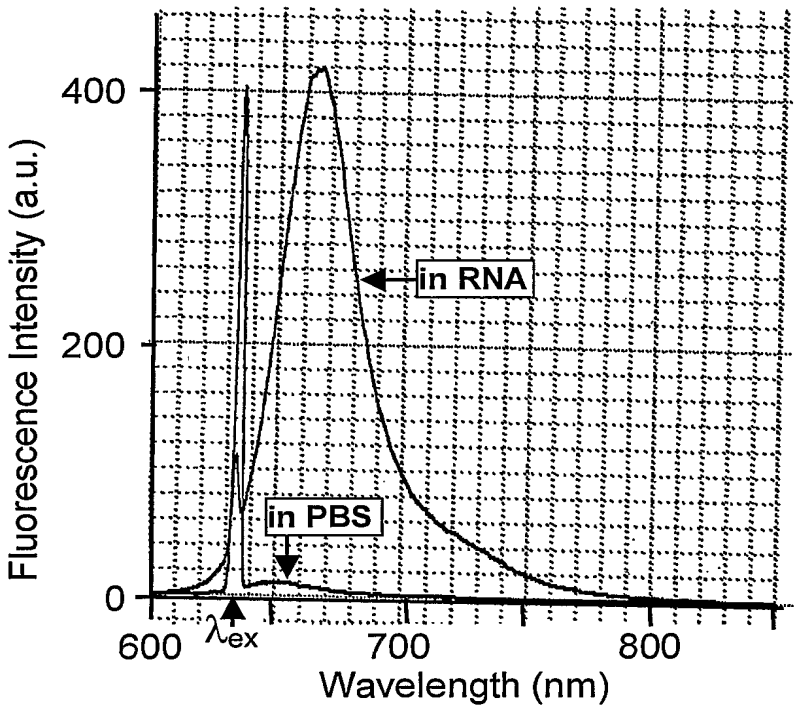

400
200
0
Fluorescence Intensity (a.u.)
in RNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9I

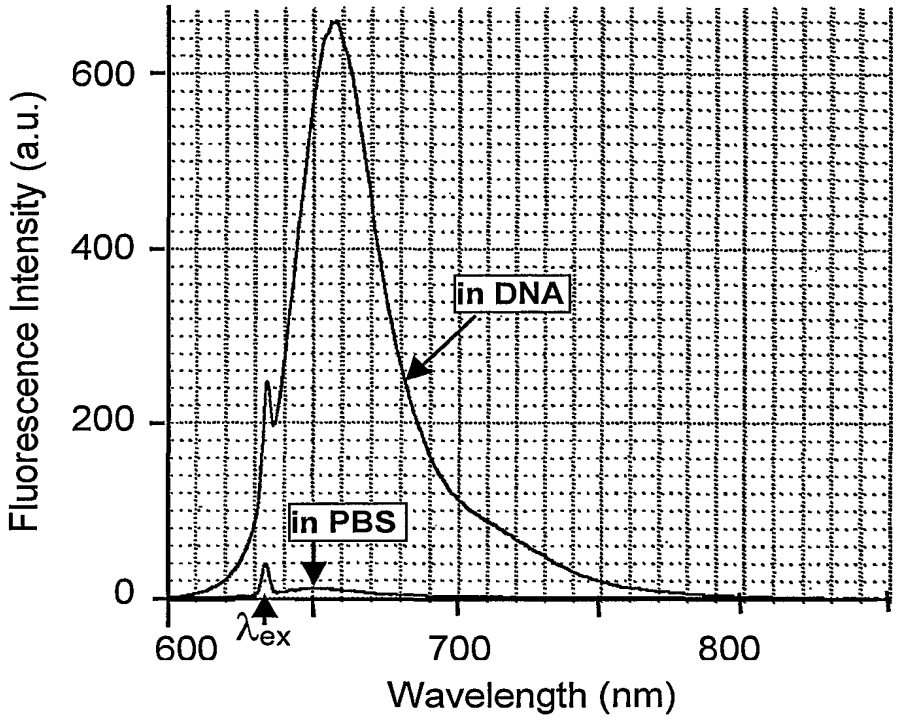

Fluorescence Intensity (a.u.)
600
400
200
0
in DNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9J

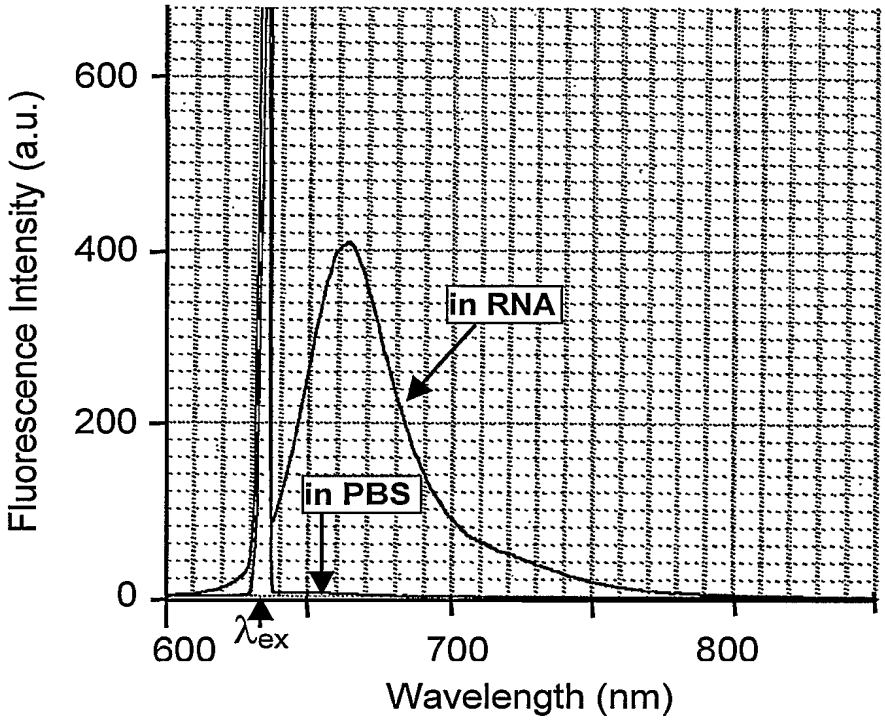

Fluorescence Intensity (a.u.)
600
400
200
0
in RNA
in PBS
600
$\lambda_{ex}$
700
800
Wavelength (nm)

FIG. 9K

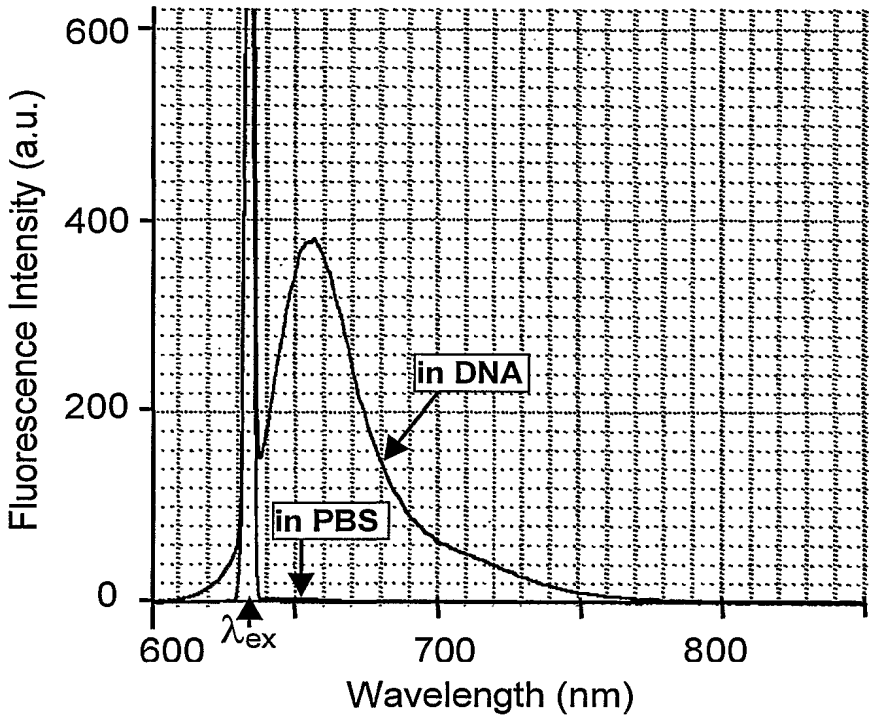

600
400
200
0
Fluorescence Intensity (a.u.)
in DNA
in PBS
λex
600
700
800
Wavelength (nm)

FIG. 9L

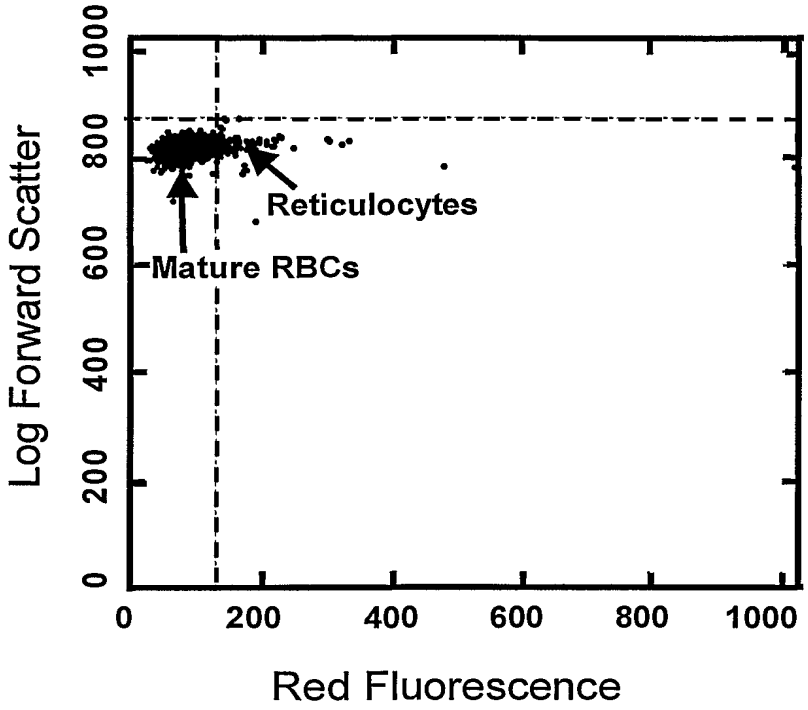



FIG. 10A

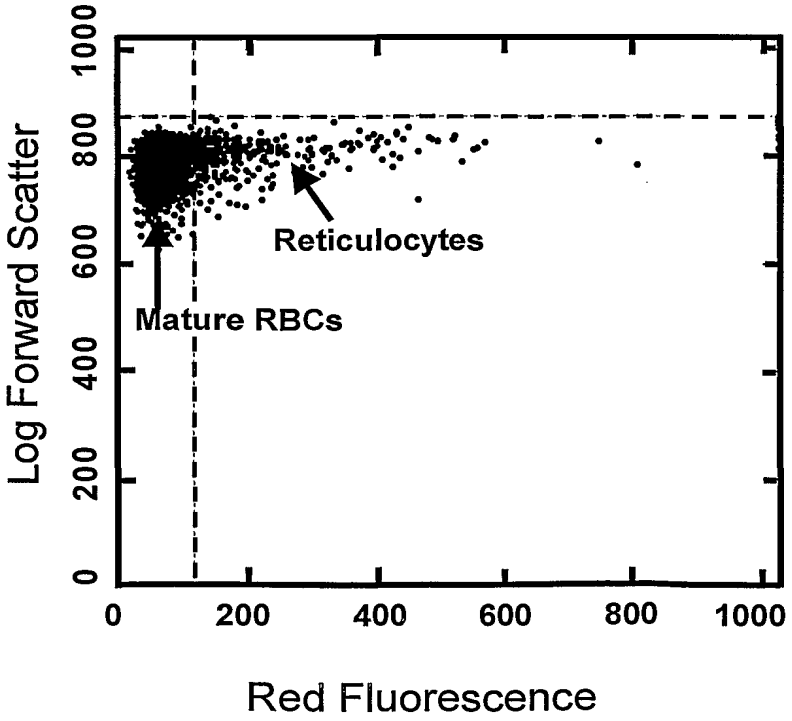



FIG. 10B

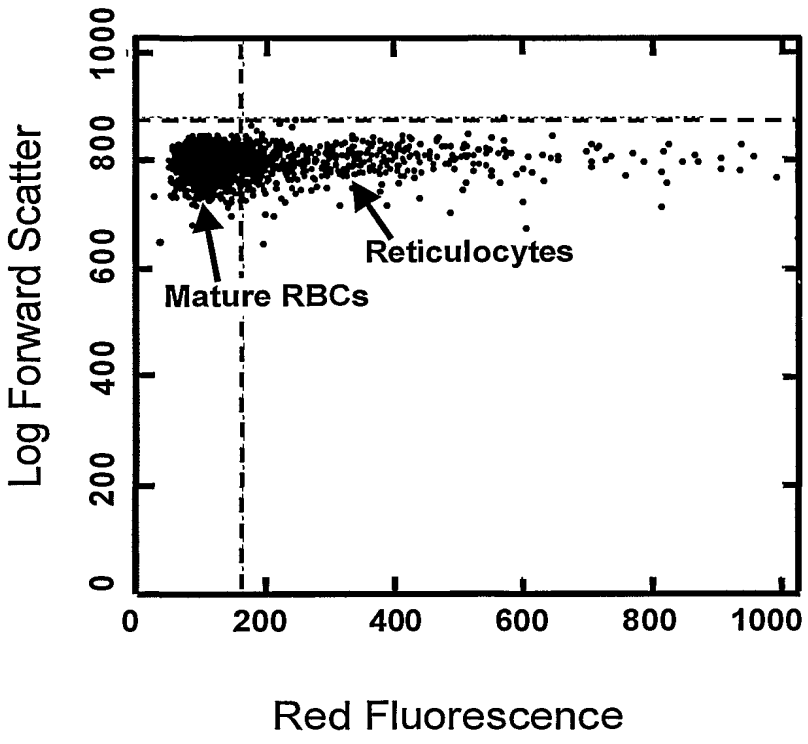

Log Forward Scatter
0
200
400
600
800
1000
Mature RBCs
Reticulocytes
Red Fluorescence

FIG. 10C

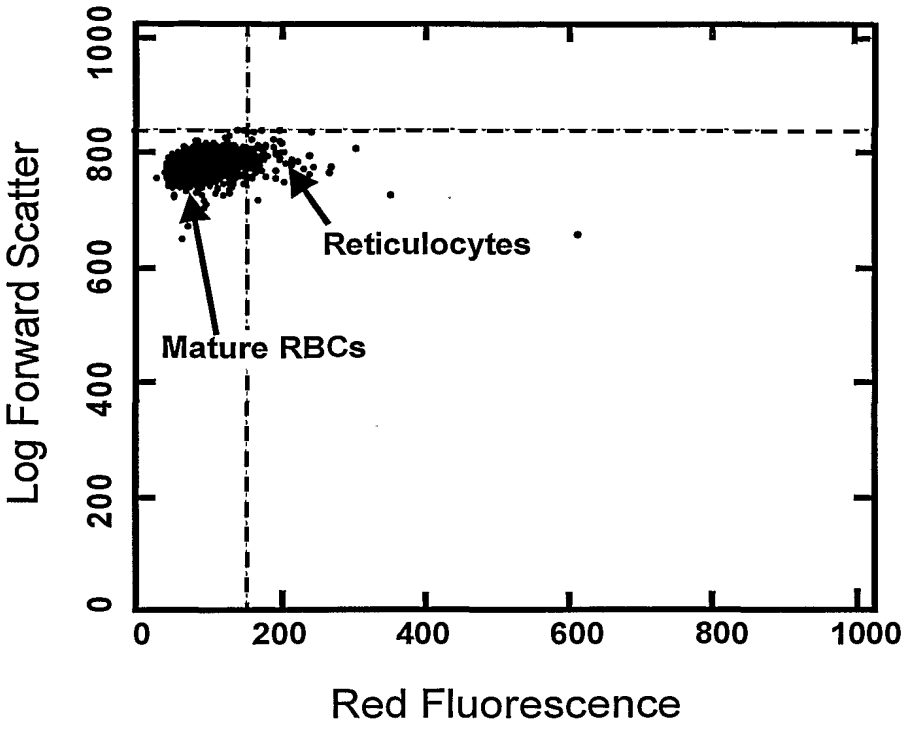



FIG. 11A

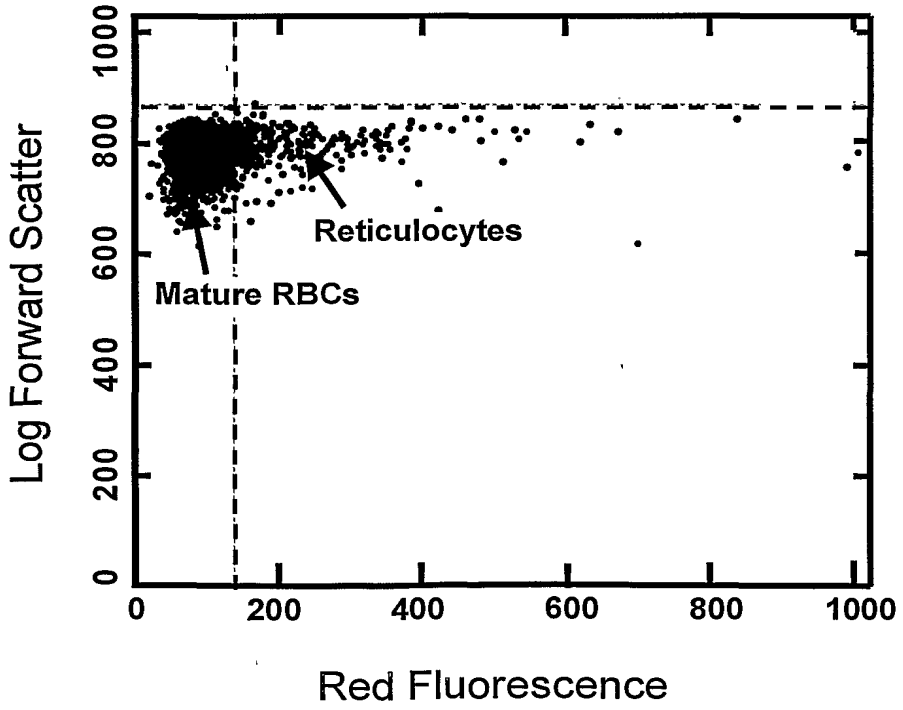



FIG. 11B

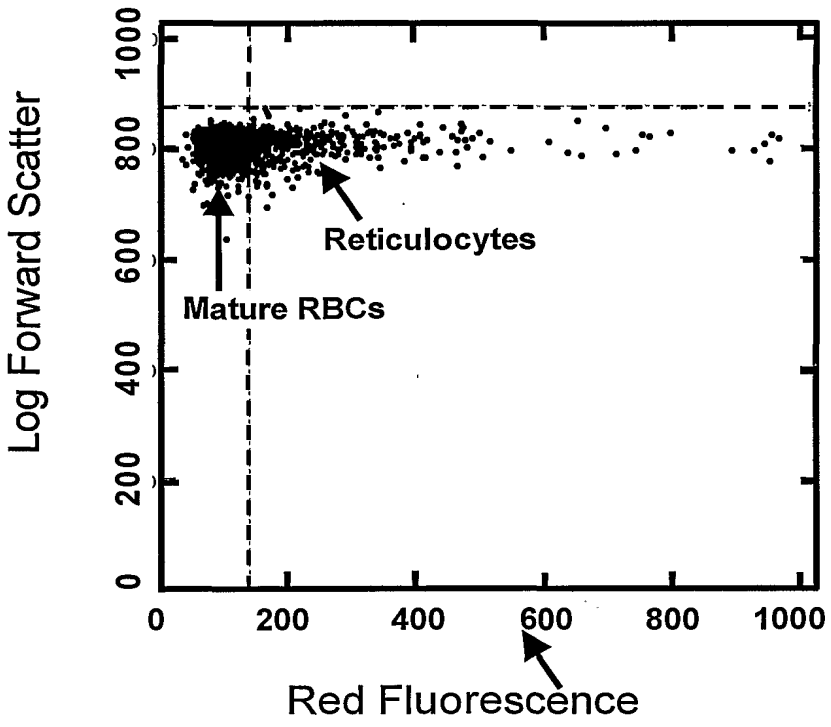

Log Forward Scatter
0
200
400
600
800
1000
Reticulocytes
Mature RBCs
0
200
400
600
800
1000
Red Fluorescence

FIG. 11C

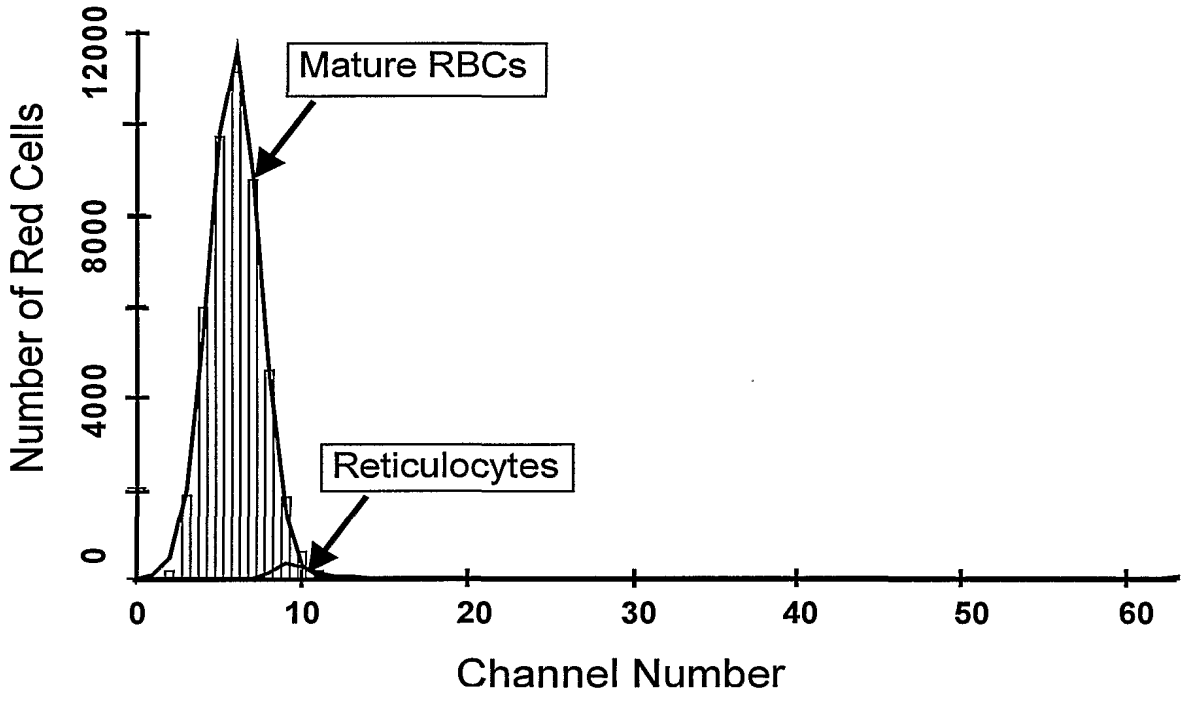

Number of Red Cells
0
4000
8000
12000
Mature RBCs
Reticulocytes
0
10
20
30
40
50
60
Channel Number

*FIG. 12A*

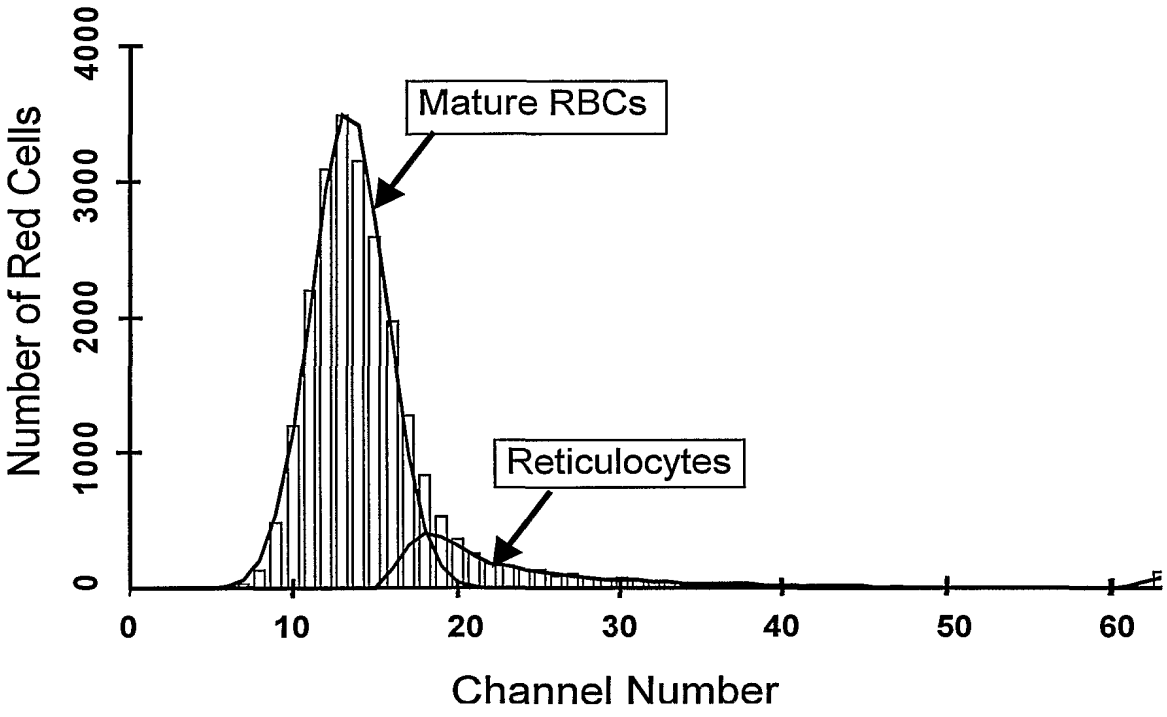

Number of Red Cells
0
1000
2000
3000
4000
Mature RBCs
Reticulocytes
0
10
20
30
40
50
60
Channel Number

*FIG. 12B*

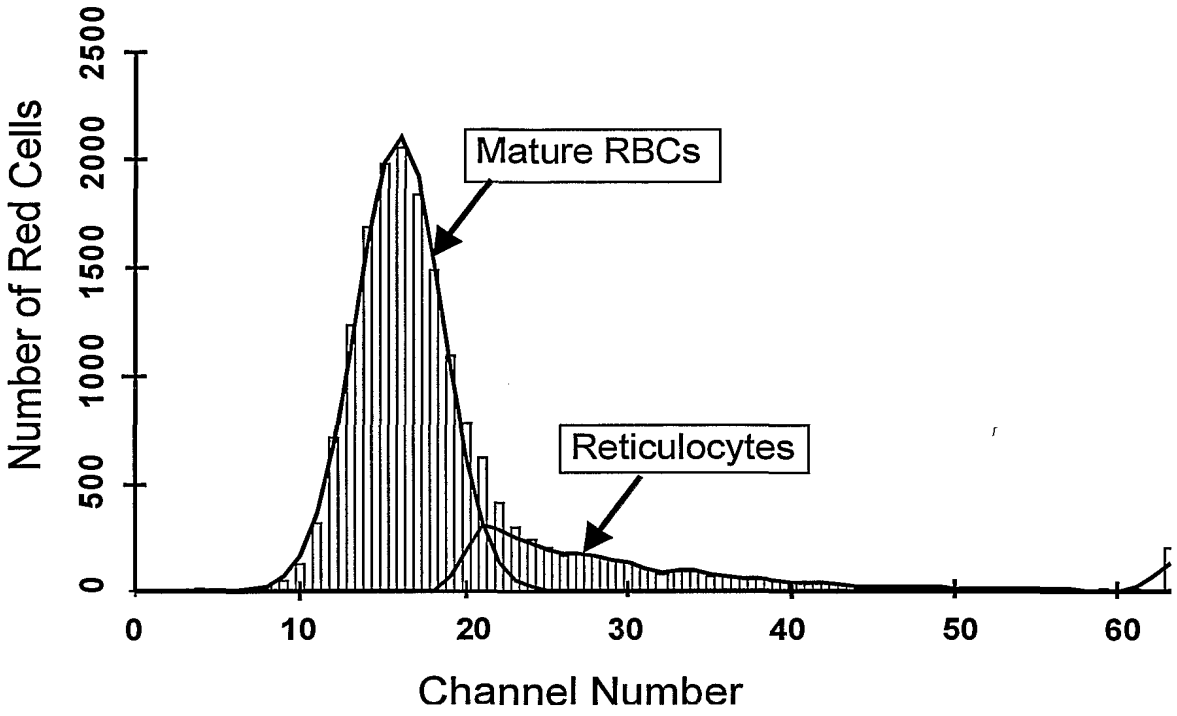

Number of Red Cells
0
500
1000
1500
2000
2500
Mature RBCs
Reticulocytes
0
10
20
30
40
50
60
Channel Number

*FIG. 12C*

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/14522

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :G01N 21/76, 33/48
US CL :436/10, 63, 164, 166, 172, 800; 252/408.1
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/8, 10, 63, 164, 166, 172, 174, 800; 252/408.1; 435/2, 29, 30, 34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields search

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN/REGISTRY, CA, ZCAPLUS, CAOLD, HCA, BIOSIS, MEDLINE
search terms: structure search, dye, stain, reticulocyte

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to clai |
|---|---|---|
| X<br>---<br>A | US 4,386,146 A (KISHINO et al) 31 May 1983, columns 2-4 and claim 1. | 1-9<br>---<br>10-72 |
| X<br>---<br>A | US 5,599,932 A (BIENIARZ et al.) 04 February 1997, Figure 19, compound no. 52, Figure 21, compound no. 56, Figure 24, compound no. 66, and Figure 26, compound no. 69. | 1, 4, 8-9<br>---<br>2-3, 5-7, 10-7 |
| A | US 5,821,127 A (AKAI et al.) 13 October 1998, column 3, lines 21-67 and column 6, lines 21-54. | 1-72 |
| A | US 5,436,134 A (HAUGLAND et al.) 25 July 1995, column 3, lines 30-68 and columns 4-5. | 1-72 |
| A | US 5,658,751 A (YUE et al.) 19 August 1997, columns 13-14. | 1-72 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|---|
| * Special categories of cited documents: | |
| "A" document defining the general state of the art which is not considered to be of particular relevance | |
| "E" earlier document published on or after the international filing date | |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | |
| "O" document referring to an oral disclosure, use, exhibition or other means | |
| "P" document published prior to the international filing date but later than the priority date claimed | |
| "T" later document published after the international filing date or prio date and not in conflict with the application but cited to understand principle or theory underlying the invention | |
| "X" document of particular relevance; the claimed invention cannot considered novel or cannot be considered to involve an inventive s when the document is taken alone | |
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